

United States as proliferation policeman

By imposing an economic ban on China in retaliation for the alleged sale of missile parts to Pakistan, the United States has done the right thing, but has gone only half-way towards the goal of limiting the spread of weapons.

THE United States has called China's bluff on nuclear arms sales to Pakistan, imposing economic sanctions against China for allegedly selling Pakistan ballistic missile technology in violation of the Missile Technology Control Regime. Taking the principled high ground, as he should, US President Bill Clinton has imposed a ban on sales worth more than \$1 billion of high technology products ranging from electronic equipment to space technology and military planes. The president has been persuaded by evidence from US intelligence agents that China, despite vigorous denials, has sold missile components to Pakistan. In particular, China is believed to have sold parts for the M-11 missile, with a range of 300 miles and capable of carrying nuclear warheads.

The seriousness of the decision should not be underestimated. It may cost US defence manufacturers as much as \$500 million a year in sales — and many of those working in the industry may lose their jobs. At a time when employment (or the lack of it) is a crucial issue in the United States, some unpopularity will fall on the president's shoulders. And although the two candidates, George Bush and Bill Clinton, more or less agreed during last year's presidential election campaign that injudicious arms sales should not be used to boost employment, Clinton is now on his own. Yet matters could get worse. If it emerges that Pakistan has been supplied with whole missiles, the administration says that present sanctions will be made still tougher.

Why has Clinton taken all these risks? The Missile Technology Control Regime, to which 23 nations have agreed in various degrees, is meant to halt the spread of nuclear weapons by stopping the spread of their delivery systems. China is not among the signatories, but agreed two years ago to adhere to its conditions, arguing all along that selling missile components to Pakistan was not a violation. But Clinton obviously has in mind the review conference of the Nuclear Non-Proliferation Treaty (NPT), due in the middle of 1995, at which the present members will be required to agree that the treaty should continue. For several months, the United States has been twisting arms, notably in Pakistan and North Korea, in the interests of non-proliferation. It is a good cause that would be made still better if the objectives were more generally understood.

But is it wise that the United States should take this whole burden on its shoulders? Would it not be better that it should take its anxieties about China's trade in missile components to the United Nations, which is in principle as competent to intervene in favour of the NPT as it is in Somalia and Bosnia?

That way, it would be possible to win the compliance of other industrialized nations in favour of a treaty whose continuation is an essential ingredient of a peaceful century ahead. And while China, as a member of the Security Council, would be able to veto proposals for coordinated sanctions, even that might help to clear the air — and rid Clinton of domestic grumbling.

The other question that arises is whether this incident will bring the United States and other industrialized governments to their senses about the sale of conventional arms, apparently to all customers. That baleful trade has strengthened with the ending of the Cold War. Instead of beating arms into ploughshares, arms suppliers are seeking to turn them into cash. That is a crazy and dangerous situation. While it is true that nuclear weapons pose the greatest threat, the most casual recollection of the Gulf War and the more recent horrors in the former Yugoslavia makes plain that vast damage that can be done by conventional weapons, themselves now capable of mass destruction. Restraint in that regard could usefully be part of the package the nuclear powers will have to try to sell to those without weapons in 1995. □

All free trade is good

President Bill Clinton must fight to win agreement to the free-trade pact with Canada and Mexico.

PRESIDENT Bill Clinton's first vacation from his still-new job may have given him the touch of reflectiveness that the United States needs in a president. That must be everybody's hope. For avoiding the pitfalls of the coming months will require the judicious blend of thought and determination whose lack has kept the president on a knife-edge in his first short spell. And what pitfalls? The immediate answer is the ambitious health-care plan, now partially unveiled and certain to be contentious and preoccupying. But there is a more urgent task: the North American Free Trade Agreement (NAFTA) between Canada, Mexico and the United States, which Clinton's predecessor signed last year and which has since been left swinging in the wind.

Clinton is understandably in a fix about NAFTA, which the Congress has only 80 days to brood about. For one thing, it was not his idea in the first place. For another, it is plain that the treaty will not pass the Congress unless the president backs it with his power and prestige. But it is also plain that



if NAFTA fails, that will seem a defeat for Clinton, meaning that he has less political capital left for the fight that matters more to him — health-care reform. But the failure of NAFTA would also be a serious setback for the United States. Both for his own sake and for that of his electors, the president should exert himself to see this measure through.

NAFTA is only a modest instrument in the liberalization of trade between Canada, Mexico and the United States. It is essentially a mutual tariff union, not an agreement of the kind typified by the Treaty of Rome which created the European Economic Community, and which legislates for the free movement of capital and people among the signatories. NAFTA will not, for example, give Mexicans the right to seek jobs in the United States without let or hindrance from the US Immigration Service; it will merely allow the goods they grow or manufacture south of the US border to enter the United States free from tariff penalties. Even so, there have been and there remain anxieties in the United States about employment, now being amplified by Mr Ross Perot; will NAFTA mean that jobs are “exported” from north to south? At the uncertain end of a sharp recession, this is the chief stumbling block to the adoption of the treaty by the US Congress.

How should Clinton tackle that objection? The temptation will be to proclaim that the anxieties provoked by NAFTA are exaggerated (which they are) and illusory (which they are not). If the agreement is effective, there will indeed be changes in the pattern of economic activity in the United States, as there will be in Canada and Mexico. But fears that US manufacturers will promptly discard past capital investments in factories to rebuild them in Mexico are misplaced. Of necessity, the most significant changes will be in sectors of the economy in which capital investment is relatively low, agriculture for example. In the longer run, of course, there will be more lasting changes. But by then, all partners in the enterprise should be more prosperous.

That is the underlying misunderstanding of NAFTA in the United States. Trade liberalization is not a zero-sum game, but one from which all partners benefit. If, as seems likely, Mexico benefits immediately from NAFTA by increasing its exports to Canada and the United States, ordinary consumers in both countries will benefit to an equal extent by the reduction of prices that will follow. In round numbers, the countable aggregate benefits of shifting patterns of trade are roughly twice their apparent magnitude. But there is more than that to say. Mexicans will have more money in their pockets, and will spend at least some of it on goods produced by their partners, while consumers in the partner states will spend what they save from cheapened purchases to increase economic activity where they are. Is that a prospect to frighten the largest and most successful economy in the world?

But the US president has more than that to tell his electors. NAFTA was made possible by the arrival of more liberal government in Mexico. For the first time since 1907 (when Arizona joined the union), the United States has an opportunity to forge enduring links with one of its Latin neighbours to the south. The political and strategic benefits of that opportunity understandably caught President George Bush’s attention. If his successor should seem to scorn them, the

influence of the United States in Central and South America will commensurably be diminished. That is one way in which the failure of NAFTA in the US Congress will be a setback for the United States. Another is that it will send a protectionist signal to Europe and Japan, all too ready to respond in kind. Clinton could avoid both difficulties by saying the right things. Let us hope he does so quickly. □

Waiting in the wings

Contrary to popular belief, the nuclear industry is not dead. It needs to be kept alive.

THE British government took part of its small stock of courage in its hands last week, and agreed that British Nuclear Fuels should be allowed to test its new reprocessing plant for nuclear fuel by running uranium oxide through the brand-new separation plant called THORP, at Sellafield in northwest England. It is a sensible decision, but has provoked environmental organizations to predictable howls of protest that the further period of consultation agreed for THORP is merely a “sham”. That is a travesty of what seems to be the truth, but is in any case misguided.

The history of THORP, which has so far cost £2,800 million, goes back 20 years, to the British decision to switch from nuclear reactors using uranium oxide rather than metal as their fuel. Given the potential economies of scale, it was decided to build a plant capable of processing oxide fuel from elsewhere, Japan and Germany in particular. Before construction began, more than a year was spent in 1977 on a public inquiry into the good sense of the project as well as its likely impact on the environment. For more than a year, the plant has been completed and its owner (itself owned by the government) has been waiting for an operating licence. But the time has gone on indecision and (now) two periods of “consultation”. The government says only that it is “minded” to give THORP a licence, but evidently not now.

This is a rotten way to run a business, even one as unpopular as a nuclear business. Several questions need deciding. First, should nuclear fuel be reprocessed to separate fissile material (uranium as well as plutonium) from fission products? That should primarily be an economic question in which the cost of generating and holding stocks of fissile material that cannot yet be used (for lack of a suitable reactor design) must be dominant. All concerned with THORP have been too secretive on this point. Second, should Britain undertake (for money) to reprocess nuclear fuel from elsewhere? Why not, if it can be done safely and profitably, especially when Britain is yearning for extra export income? Third, can the job be done as safely as British Nuclear Fuels has been claiming? It would be difficult to pretend, after the effort expended poring over the design of THORP and the storage of its products, that there is further information to be sought. Why not decide, especially when the British government must know that nuclear power stations safely designed and built are the only practical means of meeting the global warming targets it has espoused? □

Did last-minute change cause loss of \$1-billion Mars probe?

Washington. Senior officials at the US National Aeronautics and Space Administration (NASA) have revealed that loss of contact with the Mars Observer may have been caused by a last-minute decision to fire explosive valves as the probes entered orbit around Mars, instead of 11 months earlier at the launch as originally planned.

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Glenn Cunningham, Mars Observer project manager.

It was NASA's concern about shockwaves from these pyrotechnic valves — equivalent to "tapping the probe with a hammer", according to one NASA engineer — that led it to shut down communications with the Mars Observer for five minutes just over a week ago. At the end of last week, NASA had still not been able to contact the probe.

If Mars Observer is lost, the post-mortem may focus on an important change to the mission design, which is said to have been made in May 1992, just three weeks before the probe was shipped from the factory in New Jersey to Cape Canaveral in Florida for its launch.

The change affected the timing of the release of helium from a high-pressure container into two of the probe's fuel tanks, which fed the four thrusters used to manoeuvre the craft. NASA originally planned to release the helium shortly after launch, thereby keeping the fuel pressurized at around 17 bar throughout the mission. This pressure would force fuel — nitrogen tetroxide in one tank, mono methyl hydrazine in the other — into the thrusters for three small manoeuvres during flight and for the major boost in August to push the probe into orbit around Mars.

But three weeks before launch, the project team decided to pre-pressurize the fuel tanks at 17 bar before launch, and to rely on this pressure for the three small manoeuvres. The helium tank, they decided, would be

used only to pressurize the fuel just before the final boost into the Mars orbit, by releasing the four pyrotechnic valves (two on each helium pipe).

The team took this action because they were concerned that helium might leak through the regulator valves, designed to maintain fuel tank pressure at 17 bars, and cause a pressure build-up during the 11-month cruise to Mars — there had been problems on other missions and during tests.

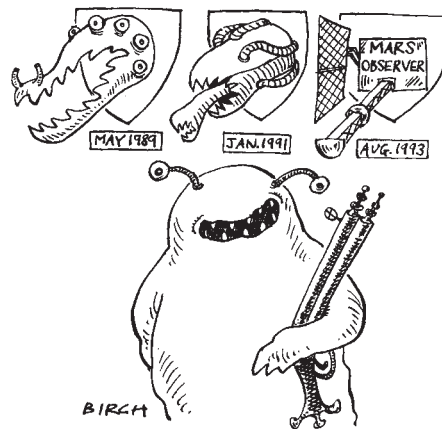
Engineers usually choose pyrotechnic valves for their reliability: unlike tap valves, they cannot jam closed, because they use a small explosive that pushes aside the metal plate that blocks fluid flow. But using pyrotechnic valves on probes such as the Mars Observer carries several risks.

■ The physical shock wave from the valve could damage sensitive components: NASA shut down the communications during last month's manoeuvre because it was concerned that the wave might cause arcing in the travelling wave tube of the probe's transmitter.

■ The explosive is detonated by a flash of electrical current of several hundred amperes. This charge cannot be earthed and must be routed back to the power source, while avoiding any detour through sensitive electronic equipment.

■ The pyro valve itself could malfunction, although this is unlikely, and possibly damage the neighbouring regulator valves that control the pressure of helium release.

Irrespective of whether any of these prob-



lems occurred, NASA — who last week named Timothy Coffey, director of research at the Naval Research Laboratory in Washington, DC, as head of the review board to investigate the loss of contact with the Mars Observer — will want to find out why the project team made a late design change that required breaking communications at such a crucial stage in the mission. Other missions to planets have sought not to break communications once they have been established in case they cannot be re-established. The Observer team will argue that they had already broken communications three times, and that there was no particular reason to anticipate failure on the fourth.

The apparent failure of the \$980-million Mars Observer mission comes at a bad time for NASA. Congress will fix the critical budget for the space station and the agency's other activities within the next few weeks. One congressional aide says that while this latest mishap is unlikely to influence veteran congressmen with fixed views on the space programme, it may turn newly elected members against NASA.

Colin Macilwain

Lost opportunities cause concern

As the mission control team at NASA's Jet Propulsion Laboratory (Pasadena, California) struggled last week to re-establish contact with the Mars Observer, scientists worldwide contemplated the possibility that years of work invested in its sophisticated experiments might have come to nothing.

"Mars Observer was planned to produce a scientific database for the whole planet, from the cellar to the attic", says Bevin French, chief programme scientist. Geophysicists and climatologists in particular hoped that data from its various instruments would improve their understanding not only of Mars but also of the Earth by providing badly needed reference points to include in models of planetary behaviour.

"What we'd really like to do", explains Fred Taylor, professor of atmospheric physics at the University of Oxford, "is to double the Earth's carbon dioxide or raise the temperature by a few degrees, and see what happens. But of course we can't do that, so we look at Mars instead." Taylor's department helped to build the infrared radiometer that would have surveyed Mars' climate from Observer. He is making sure that the department has the spare parts to build another instrument if necessary, in the hope that it might be incorporated on another mission such as NASA's Measure. "The Mars Observer science will have to be done", he says defiantly.

At Arizona State University, the 20 or so scientists and technicians doing support work for the mission's \$28-million thermal emission spectrometer, which would have mapped the minerals on Mars' surface, are dejected. "I feel fairly awful about this", says Dale Noss, a systems programmer working on software to digest data from the instrument. "A lot of us have not slept well" since contact with the probe was lost, he says. **C.M.**

UK scientists test liposome gene therapy technique

London. A group of British scientists and physicians last week began the world's first human trials of a gene therapy technique that uses liposomes rather than viruses to transfer fragments of DNA, in this case a working version of the gene whose dysfunction causes cystic fibrosis.

The news is all the more pertinent because attempts in the United States to use an attenuated adenovirus for gene therapy of cystic fibrosis (CF) patients are running into unexpected problems. In particular, a trial at the National Institutes of Health's National Heart, Lung and Blood Institute (NHLBI) has been stopped after a patient developed a lung inflammation.

Researchers are also reporting that although adenoviruses are a highly efficient way of introducing genes on their first application, the level of gene expression sometimes drops off sharply in subsequent applications.

Consequently, researchers are watching the British trial closely, because it will provide the first comparison of the safety and efficacy of both approaches. Given the vast potential market of a drug for CF, one leading US business magazine has already dubbed the competition between the two approaches as the "\$600 million horse race".

Ever since the CF gene was identified and isolated in 1989, scientists have concentrated on developing a gene replacement technique to prevent the damage that occurs when the dysfunctioning gene blocks transport of water and salt across cell membranes in the lung wall.

Earlier this year, the NIH approved the first clinical trials of gene therapy for cystic fibrosis. Ron Crystal at NHLBI is carrying

out the trial, which uses adenoviruses as vectors, whose pathogenicity has been eliminated, because they spread rapidly in the lungs.

In the British trials the genes are wrapped in liposomes. These fuse with the cell membrane and discharge the genes into the cell. The technique works in mice bred with a defective CF gene. "We are now moving from the concept to the clinical trials", says David Porteous of the Medical Research Council's (MRC) Human Genetics Unit in Edinburgh, one of the collaborators in the British trials.

Duncan Geddes, clinical director of respiratory medicine at the Royal Brompton Hospital in London, is supervising the trial which is being carried out at the hospital's National Heart and Lung Institute; the MRC and the Cystic Fibrosis Trust are funding the trial.

In the first stage, the liposomes will be administered in a nasal spray to nine young male volunteers to test if the technique is safe and effective. If it is, then the spray will be administered directly to the lungs of patients.

The British scientists say that liposomes are simpler and potentially safer than adenoviruses. "We thought that using fat-mediated entry into cells would be a milder way of doing things", says Bob Williamson, professor of biochemistry at St Mary's Hospital Medical School in London; the school has studied the genetic basis of CF since the mid-1980s.

Groups in the United States are planning similar trials, following the successful introduction of functioning CF genes into rhesus monkeys using liposomes. "We hope liposomes will prove sufficiently effective and complication-free to allow them to be

applied for the lifetime of a patient", says Robert J. Debs of the University of California, San Francisco.

Until the results of these first British and US trials of liposomes are known, there is no guarantee that they will not run into the same sort of problems as those now affecting trials of adenovirus vectors.

But commercial interest in the outcome of both types of trials is already considerable: there are 20,000 cystic fibrosis patients in the United States alone and almost 7,000 in Britain; many of them might be prepared to pay up to \$50,000 a year for a treatment that would avoid the need for gruelling and time-consuming forms of treatment. Genentech has invested \$17 million in the Maryland-based company Genvec, which Crystal set up to support his experiments. Another company, Megabios, is supporting liposome work.

British companies have shown less interest. Several big pharmaceutical companies are interested in principle, but none has yet backed clinical trials. Commercial interest has so far been restricted to the potential market for CF screening kits. **David Dickson**

Industry funding of UK universities static

London. Despite government pressure on British universities to concentrate more on wealth creation, the proportion of research funded by industry has remained static since the mid-1980s.

According to figures released last week by the Universities' Statistical Record, funding by industry increased from £78 million in the academic year 1987-88 to £120 million in 1991-92. But universities increased their total research income over the same period from £708 million to £1,137 million, leaving industry's share unchanged at around 11 per cent.

In contrast, charities doubled their funding of university research over the same period, and increased their share of the total from 15.4 to 19.2 per cent. The European Commission also increasingly supports research in British universities: such funding grew by 20 per cent between 1990-91 and 1991-92 alone, compared with a mere 2 per cent increase in research council funding over the same period.

Oxford and Cambridge reinforced their positions at the top of the total research income league, with £63.6 million and £48.5 million respectively: although the University of London's total of £314 million dwarfs both figures, this is not broken down by individual colleges.

Government support for university research — including income from the research councils and individual government departments — fell from 52 to 45 per cent of total university research between 1988-87 and 1991-92. **David Dickson**

Human Frontiers Programme wins art prize

Tokyo. Japanese researchers are pleased, but puzzled, by news that the French maker of champagnes, perfumes and designer bags, Moët Hennessy-Louis Vuitton (LVMH), is to award an art prize to the Japanese-inspired Human Frontiers Science Programme (HSFP) — for its "remarkable vision in promoting international research on the brain and biological functions."

Two of the main architects of HSFP, Akiyoshi Wada (Sagami Chemical Research Centre) and Masao Ito (Riken), will be presented with the "Trophée d'Honneur de Science pour l'Art" on 14 September in Tokyo.

Japan is pleased because it sees the award by a French company as a vindication of Wada and Ito's position in their public

row last year with Sir James Gowan, former director of HSFP, over the direction of the programme (see *Nature* 328, 527; 1992).

But Japanese researchers are still puzzled as to what a "Science pour l'Art" prize is. The explanation is that besides this one-off tribute to HSFP, LVMH every year awards two FF 100,000 prizes to individual scientists whose work has had a "direct or indirect impact on artistic creation, aesthetics, and industry": this year's winners are Sir Samuel Edwards, a theoretical physicist at the University of Cambridge for his work on polymers, and Wolfgang Helfrich, of Freie University in Berlin, for his invention of liquid crystal displays. But LVMH concedes that the links between science and art are usually tenuous. **David Swinbanks**

Combined science courses halt declining student interest

London. The introduction of combined science courses, replacing the traditional emphasis on the three separate disciplines of physics, chemistry and biology, appears to have stemmed a declining interest in science among British school students, at least in terms of overall numbers.

But the lack of any significant increase in interest in science, particularly when combined with a continued drop in the proportion of students studying science subjects at Advanced (A)-level, is putting pressure on the government to take stronger steps to increase the country's production of trained scientists and engineers.

General Certificate of Secondary Education (GCSE) results published last week show that the number of 16-year-olds taking the traditional subjects is continuing to decline. The biggest drop was in physics, where the number of candidates fell by 16.4 per cent compared to 1992;

in chemistry and biology, the drop was 13.7 and 12.2 per cent respectively.

In contrast, the number sitting the combined science papers — which count as two separate GCSE passes, and are now, as a result of the introduction of the national curriculum, taken by almost three times as many candidates as those taking single science subjects — increased by 3.7 per cent.

Taking account of the falling number of 16-year-olds in the population, the net result is that the total number of GCSE passes has remained virtually constant. Furthermore, the proportion of candidates achieving the top three grades has increased for those taking both single subjects and the combined science course.

The GCSE results have been published a week after those for school students taking A-levels. These revealed a more worrying picture, and in particular a significant drop for the fourth successive year in the number

of students taking individual subjects. For example, the number of candidates for physics fell by 9.6 per cent (and for mathematics by 10.6 per cent).

Such figures are not as dramatic as they appear at first sight, as they do not take into account the falling number of 18-year-olds in the population. When this is done, the proportion of this age group taking science A-level has remain roughly constant over the past five years.

Nevertheless one immediate result of the A-level results is to put pressure on the government to introduce new steps to encourage students to follow science rather than arts subjects. Although universities have, with government backing, been increasing the number of science courses on offer, the supply still outstrips demand.

At the end of last week, for example, almost all British universities were still advertising vacant places on courses in physics, chemistry and biology, in contrast to humanities courses, particularly in English and modern languages, from which large numbers of well-qualified applicants have been turned away.

This situation has been encouraging some universities to offer places on conversion courses to students who had been unsuccessful in their bids for places on arts courses. Supporters of these courses claim that they play an important role in promoting a general awareness of science among those planning to enter industry and commerce. But many scientists remain sceptical, claiming that they could become more a way of filling empty lecture halls than of producing creative researchers.

Some organizations claim to see a silver lining in the A-level results. In particular, they suggest that the publicity being given to the large number of vacancies on science courses may encourage some students currently planning to take arts subjects as A-level to switch streams.

"The results are also going to make universities look more closely at how they match the courses they are providing to the changing qualifications of students applying for them", says Cathy Wilson, education officer of the Institute of Physics.

Others, however, warn that the continuing reluctance of students to choose A-levels that will equip them to read science subjects at university reflects a continued devaluation of science by both government and industry, reflected both in the continued lack of employment opportunities for science graduates and their relatively low salaries compared to other professions.

"Even though we are seeing broad support for the changes being introduced at GCSE, the overall figures are not very encouraging, and I worry about the whole climate in which children are not being actively encouraged to pursue careers in science", says David Moore, general secretary of the Association for Science Education.

Backlash threatens biomedicine

Keele, Staffordshire. Britain's biomedical scientists were warned this week that public disillusionment with the benefits of "high-tech" medicine could trigger a backlash against biomedical research.

The warning came from Sir David Weatherall, regius professor of medicine at the University of Oxford, in his presidential address to the British Association for the Advancement of Science, held at the University of Keele.

Weatherall said that the public is becoming less enthusiastic about scientific approaches to disease and is turning to alternative approaches. The whole ethos of modern medical practice is being challenged, he said. "While much of this new thinking stems from a genuine wish to improve the quality of patient care in the shortest possible time, there is a danger that it will undermine the role of the basic medical sciences, which have to take a longer-term view of the control of disease."

He singled out genetic engineering as one field in which the public is often misled about the speed with which potential benefits are likely to emerge.

"Claims by molecular biologists that,

within the next five to ten years, we will be able to identify the major genes involved and somehow alter our susceptibility to our common killers, are naive and misleading, based as they are on a complete misunderstanding of the enormous complexity of these diseases and those who suffer from them."

Each breakthrough in molecular sciences promises to revolutionize the treatment of a particular disease, but so far has had little impact on our daily lives.

One result has been an enormous increase in the growth of alternative medicine, said Weatherall. Another has been increasing pressure on medical schools to increase their emphasis on communication skills and social science at the expense of basic biological sciences, "It is not surprising, therefore, that our university clinical and basic science research departments are feeling uneasy."

Reaction against the way in which medical practice has evolved in recent years has created a feeling that high-technology medicine is dehumanizing, and that more attention needs to be paid to preventive medicine. "But because these changes are occurring at a time when society is disillusioned with science, and when support for medical research is so limited, there is a genuine danger that the pendulum may swing too far, and that, in our new customer-driven approach to medical research, the vital role of the basic medical sciences may be neglected."

David Dickson



Sir David Weatherall

Russia to let Norwegians visit nuclear graveyard

Stavanger. The Russian government last week gave permission for a joint team of Russian and Norwegian scientists to examine nuclear reactors dumped at sea by the Soviet navy in the 1960s and 1970s off the Arctic island of Novaya Zemlya.

This apparent breakthrough in environmental diplomacy was announced following a meeting in Stavanger between the Norwegian environment minister, Thorbjørn Berntsen, and Russia's first deputy minister for environmental protection, Alexei Poryadin. Both men had been attending a conference on environmental protection.

The Norwegians have in particular expressed concern that pollution of the Kara Sea as a result of the dumping may contaminate the Barents Sea to the west, which holds enormous fish stocks.

Preparations had been completed several months ago for a joint expedition to the dumping site. Until now, however, Russian military authorities had barred access to the area. An expedition last summer took sand and water samples from the Kara Sea, east of Novaya Zemlya, but was prevented from passing within 12 nautical miles of the island.

Following a change of heart in Moscow, the expedition is now expected to leave Kerkenes, on the Norwegian-Russian border, this week. It will last for four weeks, and its equipment includes remote-operated vehicles for taking samples and photographs on the seabed.

For many years, Russian authorities denied that any reactors had been dumped at sea. But in October 1992 President Boris Yeltsin ordered an investigation which opened some of the military records and revealed the full extent of the navy's activities.

It is now thought that 13 nuclear submarine reactors were dumped in the region, as well as three reactors from the nuclear-powered ice breaker *Lenin*, operated by the Murmansk Shipping Company. Particular concern has been generated by the fact that six of the reactors were apparently dumped with their fuel rods in place.

The results from last year's expedition, made public in Kerkenes last month, show that radioactivity has been contained within the dumping sites. Indeed, more than half the radioactivity detected in the Kara Sea was traced to the Sellafield nuclear plant in England. Other sources include Soviet nuclear testing sites.

"The overall radiological situation is good" says Jan Thompson, director general for international and polar affairs at the Norwegian Ministry of Environment. "It is

the potential risk that is of concern."

The new expedition will visit two of the three bays on Novaya Zemlya's east coast where reactors were dumped. Dumping in Stepovo Bay was at a depth of only 25 metres; in Sivolvky Bay it was between 50 and 135 metres. A third site, in a 380-metre trench of the Kara Sea, will also be investigated by the expedition, but the remote-operated vehicles cannot be used at this depth.

Access to the most severely affected site, Abrosimov Bay, is still being denied by Moscow. But Norwegian scientists are optimistic that the ban may only be temporary, particularly given the incentive of access to Western funds and support for other environmental projects. "The Russian government has made a firm commitment to map

out all the dumping sites between now and 1995", says Thompson. "We have reason to believe the Russians will be interested in continuing the work."

Knut Eric Nilsen, of the Bellona Foundation, an environmental group that gathered information about the dumping, has welcomed the change of heart in Moscow, but remains cautious about the outcome. "We have to see if the inspections actually take place", he says. "The most important thing is to know what has been dumped so that a priority list [for action] can be drawn up."

The involvement of Western scientists and officials in the expedition is being seen as vital for the project's credibility. Its supporters include the Norwegian Radiological Protection Authority, the International Atomic Energy Agency and the European Commission in Brussels.

Isotopic analysis will be carried out by the Agricultural University in Åa, near Oslo, and scientists from the Institute of Marine Research at Bergen will also be involved in assessing the current risks.

Julian Rose

MITI to boost venture businesses

Tokyo. The Japanese Ministry of International Trade and Industry (MITI) wants to revitalize its efforts to promote venture businesses in Japan and their research and development activities with a series of new incentives included in the ministry's budget proposals for next fiscal year. But the Ministry of Finance will probably strongly resist some of MITI's plans because of the poor state of the nation's finances.

Venture businesses are rare in Japan. There are, for example, no equivalents of biotechnology companies such as Genentech in the United States. Instead, biotechnology research is done by large chemical and pharmaceutical companies and tends to be unadventurous.

Japan lacks venture capital, and most comes from subsidiaries of banks and security companies which are not prepared to take high risk. It also lacks entrepreneurs prepared to create venture companies, unlike the United States where many university and company researchers have set up such businesses to commercialize their ideas.

MITI has long tried to encourage the creation of venture businesses, but with little success. In 1975, the ministry's machinery and information industries bureau established the Venture Enterprise Center (VEC). This provides low-interest loans of up to ¥80 million (\$770,000) to small companies. VEC funds come from the profits of gambling on bicycle races, which are controlled by the ministry (see *Nature* 363, 386; 1993).

But VEC has supported a limited range and type of venture businesses, and is

strongly influenced by the interests of the bureau and the small committee of researchers and industrialists that screen the applications. It has, for example, provided very little support for biotechnology ventures.

MITI and VEC officials will go on a study tour of the United States next week, including visits to biotech companies. And MITI officials hope to breathe new life into the enterprise by increasing its funds so that it can take greater risks, and by appointing new members to the screening committee.

Another wing of MITI, the Small and Medium Size Enterprise Agency, also has new plans. It hopes to increase its budget for subsidies to small companies for technology development from ¥1.3 billion this fiscal year to ¥1.8 billion (\$17 million) in fiscal 1994. But this will be difficult. The Ministry of Finance is trying to freeze spending next year because of a shortfall in tax revenues caused by the recession.

The agency also wants three Small Business Investment Companies (SBIC, in Tokyo, Nagoya and Osaka) that are under its jurisdiction to be allowed to establish tax-free reserve funds of several billion yen to create new small companies or support young companies. With such funds, the SBICs could avoid paying 37.5 per cent corporate tax on any losses they might make, which would give them greater incentive to invest in more risky ventures.

But the Ministry of Finance will also strongly resist this proposal because it would reduce future tax revenue. The Ministry will decide on MITI's proposals at the end of the year.

David Swinbanks

MITI and STA avoid squeeze on spending

Tokyo Japan's Ministry of Finance has taken advantage of the recent change of government to try to clamp down on spending on science next fiscal year. But the Ministry of International Trade and Industry (MITI) and the Science and Technology Agency (STA) have avoided the squeeze and include significant new appropriations in their budget requests for 1994 submitted on Tuesday, 31 August.

Days after the new Prime Minister Morihiro Hosokawa took power, the Ministry of Finance insisted that a new category of budget established last year by the former ruling Liberal Democratic Party (LDP) to support academic research should be abolished. The demise of the LDP gave the ministry the opportunity to make this move.

The new fund in theory added ¥110 billion (about \$1 billion) to the budgets of all science-related ministries and agencies. In practice, it merely reduced the amount by which the budget for general operating expenditures must decrease each year under a policy of fiscal restraint (see *Nature* **358**, 530; 1993). Nevertheless, it did give the science-related ministries and agencies room to win some of the biggest increases in their overall budgets in many years.

Abolition of the fund has been a "very big disadvantage" in budget negotiations, says Tadatsuma Koda, director general of the general coordination department of the Agency of Industrial Science and Technology (AIST), MITI's main research arm. AIST's budget request for its general account increases by only 2.7 per cent, compared with a 4.6 per cent increase this fiscal year, and this request will be trimmed in negotiations with the finance ministry before the budget is finalized at the end of December.

MITI is getting around the squeeze by boosting a separate special account derived from taxes on fossil fuels and electricity. With a large increase in this account, MITI hopes to get an overall increase of 7.5 per cent in its research and development budget to ¥302 billion (\$2.9 billion). Most of the extra money is going into projects related to development of environment friendly technology, such as the 'New Sunshine Programme' and the Research Institute of Innovative Technology for the Earth (RITE).

The STA, which is requesting an increase of 6.1 per cent to ¥617 billion, has also avoided the worst of the squeeze because a large portion of its budget, for example for space and nuclear power, is classified as "investment" and can be increased by 5 per cent under Ministry of Finance rules. Furthermore, STA's commitments to international projects such as

the US Space Station and the International Thermonuclear Experimental Reactor are not subject to such severe budget restrictions.

A key component of STA's budget is a request for ¥1.1 billion to establish an inter-ministry backbone computer network linking national research institutes and universities (see *Nature* **363**, 575; 1993). The backbone running from Tsukuba science city through Tokyo to the Osaka – Kyoto area will have a capacity of 6 megabits-per-second, more than 10 times the capacity of existing lines, and will link 40 – 50 national institutes of

numerous ministries and agencies, including even one of the Ministry of finance and another of the Police Agency. The backbone will also be connected to the SINET backbone that links universities run by the Ministry of Education, Science and Culture and to the 45 megabit network of the National Science Foundation in the United States.

STA also requests a doubling in its budget for 'centres of excellence' from ¥1.2 billion to ¥2.4 billion to allow three more centres to be chosen next year (see *Nature* **364**, 660; 1993).

David Swinbanks

Science jamboree in transition

Keele. The British Association for the Advancement of Science (BA) seems to be having a good annual meeting this year, even though holed up and isolated on this 1960s campus, a grand country house whose surrounding park has been half-filled with teaching blocks and student dormitories in an architectural style that, except for a few touches of new brutalism, is a monument to an earlier age of optimism.

The annual meeting, trendily renamed a "science festival", used to be the BA's sole activity and only contact with its members. Now it has an annual budget of £1 million (mostly from grants and commissions), and has embarked on novel ventures such as a meeting earlier this year intended (now altogether successfully) to confront financial institutions of the City of London with scientists' complaints that short-term money calculations are the death of innovation.

The most important ingredient in the BA's budget is a government grant of £140,000 (this year), laundered through the Royal Society; without that, other grants (notably £50,000 a year from the Wellcome Trust) would be raised only with greater difficulty. But Peter Briggs, a more hard-headed secretary than the BA has had for decades, recognizes that the organization needs more paying members; there are only 1,700 at present, of whom a large number bought life memberships at earlier bargain prices.

The association, earlier this week, was also looking for some tangible further encouragement when Mr William Waldegrave, the government minister with responsibility for science and technology, who would appear at Keele later in the week. (This visit by a science minister to the BA's annual meeting is unprecedented, but so, apart from a brief spell in the early 1960s, is Waldegrave's post.)

The annual meeting has been modestly transformed from the pattern of the distant past. This year as for some past, the occasion has been used by organizations other than the BA's own specialized section to present symposia, to the benefit of all. Thus Keele is the occasion of the Fifth European Skeptics' Conference (that of the people who have no time for the paranormal except for what they spend denouncing it), a visionary look forward organized by the Royal Society's Committee on the Public Understanding of Science and a similar exercise in the field of health organized by Glaxo, the pharmaceutical manufacturer.

Yet the oddly miscellaneous character of the programme persists, with the case for hydrogen as a road transport fuel (Professor James Randle, University of Birmingham), the conservation of tropical rain forests (a chorus of speakers), the continuing shortage of specialist teachers in British schools (Professor Alan Smithers, University of Manchester) and the case for regarding financial speculators as moral agents of the financial system rather than as immoral parasites on it (Sir Samuel Brittan, *Financial Times*). The last will be supported by an ex-chancellor of the exchequer, Lord Lawson, later in the week.

As always, to judge from a small sample earlier this week, individual presentations tend to overstray the allotted time, leaving too little for discussion. And such as there is tends to be muted by diffidence and gentility. That may be another reason for applauding the BA's ambition to concentrate, in the future, on taking its annual message to the big cities, where there will be more day visitors (who will also contribute to the association's funds). It seems, as always, a pity that so much scholarly entertainment should be so confined.

John Maddox

Continuing confusion

SIR — Beck¹ claims that the sex ratio (proportion of males at birth) of artists' offspring is high. Mueller² offers the Trivers-Willard hypothesis³, an 'appropriate theoretical framework' to account for such a finding.

But even if Beck is right, I suggest that Mueller's judgement is premature. Mammalian⁴ (and among them, human⁵) sex ratios depend on many variables. Some of this variation is consistent with the Trivers-Willard hypothesis and some is not. For instance, some human diseases (such as prostatic cancer⁶ and hepatitis B⁷) are associated with the production of male, not female, offspring. But it is not yet possible to tell whether any given sex ratio bias is adaptive or the consequence of some physiological constraint.

I have suggested that the problem should best be tackled by examining the proximate mechanisms underlying sex ratio variations⁸ which are likely to be far fewer than the number of variables with which sex ratio has been found to vary. So it would seem sensible to ask whether these mechanisms (rather than the variables) behave in conformity with adaptive hypotheses. I have proposed that the sex ratio of mammalian offspring is partially controlled by parental gonadotrophin and steroid hormone levels at the time of conception⁹. In men, illness of various sorts is associated with high gonadotrophin and low testosterone levels¹⁰ and, *ex hypothesi*, with low offspring sex ratios (thus presumably conforming with the Trivers-Willard hypothesis). I have also given grounds for suggesting that people suffering from, or destined to suffer from, prostatic cancer or hepatitis B have high testosterone levels¹¹. If this is correct, the high sex ratios of the offspring of these people may come to be viewed as a result of a physiological constraint rather than evidence against Trivers and Willard. But such matters should be settled before elevating their hypothesis into a 'theoretical framework'.

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SIR — I respond to Ulrich Mueller's comment on social status and sex (ratio of offspring), based on an examination of biographical compilations². The authors are presumably interested in the biological phenomenon of sex ratio at (live) birth. The representation of children in compilations such as *Who's Who* is a socially stressed report that is subject to a variety of distortions: reporting of perinatal mortality, illegitimacy, children of plural mar-

riages, adoption and repudiation: all these are likely to be biased by social class and by sex. The data given can hardly be accepted at face value. If they were to be, we would then have to remove confounding with variables such as parity and age of mother, as well as differential prenatal mortality that may be associated with infection, malnutrition and other socially correlated causes.

Unfortunately, it is difficult to find large datasets organized by biological family relationship. The large population census datasets are acquired by household rather than family, and are beset with many errors in statistical detail¹².

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Gaining time

SIR — The leading article in *News and Views* on "Competition and the death of science" (*Nature* **363**, 667;1993) raises important points about the dangers of competition. It also illustrates another important point about the funding of science in general. The allocation of large sums of money to some fields is a good illustration of the law of diminishing returns and is probably not an efficient use of resources (including human capital).

There are fields in which advances are strongly limited by funding. As a result, it is often not the best scientists (nor the best ideas) that get funded but those willing to switch to less basic but more easily funded projects. We are forced to consider not whether an experiment tests an important hypothesis but whether it will attract funding. An increase in funding could have a major impact in such fields, where advances may now have to wait 5 or 10 years because of funding problems. It is not that we do not have clear testable hypotheses or the knowledge and techniques to test them. Needless to say, this results in considerable frustration and depression among the workers in such fields. Both society and the individuals lose.

In contrast, there are fields where if one person is not funded, the experiment will probably be completed in another laboratory within a few months. Large sums of money are thus seemingly being spent to advance science by a short time. Perhaps more balanced funding would bring about a more efficient use of resources.

Research councils should therefore consider developing a system which will include the following simple question. "If we fund a project in field A (assuming the referees agree that it has a clear, logical and testable goal) does it have the potential to advance the field by a few months or by a few years or a decade?" I suggest that money be invested where the largest gains in time are probable.

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Polluter pays

SIR — Your suggestion in the leading article "Environmental protection or imperialism?" (*Nature* **363**, 657;1993) that underdeveloped countries may not be able to afford environmental protection might have some merit with respect to goods produced for home consumption, but there is no reason why affluent customers should not pay the full price of production, including the price of the safe disposal of the pollutants produced in the process of manufacture. Neither is there any reason why pollution control should be more expensive in undeveloped than in developed countries, nor why multinational corporations that depend on worldwide markets should not (through prices charged to their customers) protect the environment wherever they are.

Your gratuitous accusation that we in the United States are motivated more by fear of the loss of jobs than by concern for the environment is similar to my accusing you of caring more for the profits of multinational corporations than for the environment — but I wouldn't say a thing like that.

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Now we know

SIR — Although a British MP once pronounced that dogs do not have DNA, can there be a single member of the public that does not know that dinosaurs had DNA?

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Marrying computers with biology

An interesting symposium last month suggests that computation and molecular biology, already interdependent, are about to become inextricably linked.

Waterville Valley. Ski resorts like this can still be alive in the summer, as Aspen in Colorado has proved. This hilly (rather than mountainous) patch of central New Hampshire is a poor man's Aspen in the winter: the quality of the snow leaves experienced skiers scornful (and, until last winter, there had not been much of it for three seasons). But this summer the condos (short for 'condominiums') have been brought to life not just by trade conventions but by the Third International Symposium and Workshop on Macromolecules, Genes and Computers.

What happens when you throw together more than a hundred each of molecular biologists and computer scientists for the best part of a week? That, of course, is more than an experiment in sociology: these days, the two groups have tangible things to say to each other. That they would seems to have been why Professor Temple F. Smith of Boston University organized the first of these events six years ago. In the general opinion of those at this year's meeting, he ought to begin now to organize the next occasion in three years, or even sooner. Most of those present seem to have been stimulated by this year's proceedings (17–22 August).

Computer people and molecular biologists talk to each other on several different levels. There is, for example, the house-keeping of molecular biology — the management of databases such as those accumulating the nucleotide sequences of genes and even genomes, the amino-acid sequences of protein molecules and the atomic coordinates of the macromolecules determined by X-ray and other techniques. But computers are increasingly one of the means by which problems in molecular biology may be solved, perhaps by the intelligent display of information (as with molecular models) or the discovery of the structure of a molecule by molecular dynamics or some other technique. But the ambitious among the computer people look further ahead, to the point at which hardware, perhaps in the form of neural networks or some other embodiment of artificial intelligence, will answer and perhaps even ask questions of a more generally conceptual character.

What follows is not a formal report of this stimulating occasion, but an impression of it. And the outstanding ingredient of that must be the way in which the basement room given over to the workstations used to demonstrate the software people had brought with them to the party seemed always to be crowded with inquisitive potential users. Interestingly, the bulk of those with soft-

ware to show off are people with a background in biology, but there were also refugees from fields such as astrophysics where, for one reason or another, financial support is hard to come by. One purist of a computer scientist used the word "amateurs", but softly.

The amateurs, however, have a down-to-earth appreciation of what their potential users need. Making genetic maps available on notebook computers typifies a common assumption that the user molecular biologist will not be separated from his or her data for any length of time. The general willingness to let others have copies of interesting software is impressive. So is the generality of the ambition that the better projects will be taken up by professional software houses. Indeed, that rather than what potential users say, seems to be the touchstone of quality.

But many of them also share a vague sense of disappointment that most of the users are still asking relatively unsophisticated questions of the data accumulating in the databanks. Searching through a genome, or the parts of it already stored as data, for the occurrence of a predetermined nucleotide sequence, is easy enough, even if it takes a little time. But at least one computer buff looks forward to software designed to annotate the nucleotide sequences submitted to the databanks which will deliver to the author of the data a comprehensive statement of their biological interest.

But is not most of any genome junk, in any case? Sydney Brenner, putting the case for the study of the genome of the Japanese puffer-fish as the most compact vertebrate genome and thus that best suited as an intermediate between those of the nematode and the human being, took it for granted that most of the junk is without meaning. But some of the arresting work described at the meeting showed that regulatory sequences for specific genes may be found tens of thousands of kilobases from the genes whose activity they regulate, not only upstream of them but also downstream. But if at least some genes are not much larger than the sequences that specify the structure of the proteins transcribed from them, the prospects for the automatic readout by machine of the properties of a genetic sequence are evidently diminished. In other words, molecular biologists would be asking more sophisticated questions if their field were not at such an early stage in its development.

So much is also clear from the famous protein-folding problem, on which a substantial subset of the computer people have lavished effort. The most effective compu-

ter-aided folding on show here has been that in which the unknown structure of a protein is guided by the homology there may be between its sequence of amino acids and that of a protein whose structure is stored in one of the databases. *De novo* folding by machine remains an uncertain business.

Luckily, there seems to have been more confident progress in the use of packages of molecular interactions, and of the very substantial computing engines, to predict the structures of RNA molecules specified simply by their sequences. But in both cases, the interactions of the specified molecules with their mostly aqueous surroundings is accounted for by supposing the exterior to be homogenous. Could it be for that reason that the computers have so far had so little to say about the functioning of macromolecules with relatively specific functions, as enzymes or as ribozymes?

Issues concerning RNA kept cropping up at the meeting, notably because of enthusiasm for the notion that random assemblages of RNA molecules may have been the entities from which life emerged. Indeed, Stuart Kauffman from the Sante Fe Institute provided an account of how, by arranging to assemble a random collection of building blocks of macromolecules "in a pot", it should be possible to make and identify novel biological chemicals. But that was bettered by an account of an experiment at the Massachusetts General Hospital in which a collection of random RNA molecules has been induced, under external selection pressure, to evolve a more effective ligase than that possessed by the cells from which the random molecules were derived.

These, of course, are the hints at how life may have begun that send the computer people rushing to their notepads. Although it appears that there is already a computer game on the toy market that simulates evolution, the interest in building really sophisticated models is powerful, and unlikely to be subdued by uncertainties about the parameters. The difficulty of modelling the mix of carbon compounds recognized by radioastronomers in interstellar space may be a more salutary lesson.

Meanwhile, there seems no doubt that the marriage Temple Smith effected with some difficulty six years ago has come to stay. It is not just that each partner recognizes the need of the other, but that each also sees opportunities that could not be accomplished in isolation. It is an easy guess that the fourth workshop will be even more instructive.

John Maddox

Anionic electrons in electrides

James L. Dye

ELECTRIDES are curious ionic compounds: the electrons, rather than being fully delocalized or else an adjunct to a particular atom or molecule, apparently sit in the crystal lattice for all the world as if they were anions. On page 39 of this issue, Singh *et al.*¹ provide strong theoretical confirmation that the electrons are indeed trapped at the anionic site, and not, as recent interpretations of optical and NMR data might suggest², localized close to the cation.

Electrides have the general formula $M^+(L)_n e^-$, in which M^+ is an alkali metal cation, L is an organic molecule (Fig. 1) that tends to sequester the cation in a 'cage' when $n = 1$ or between the halves of a 'sandwich' when $n = 2$, and e^- represents the trapped electron. Electrides form when alkali metals interact with an organic complexant to release the outer s electrons and form large complexed alkali

metal cations. These cations (which contain Li^+ , Na^+ , K^+ , Rb^+ or Cs^+) pack together to form a crystalline solid and leave vacancies of diameter 4–5 Å at which the released electrons are presumably trapped. The first crystalline electride was synthesized in 1983 and its crystal structure was determined in 1986. Four electrides now have known crystal structures, all of which contain apparently 'empty' cavities that can trap one³ or two⁴ electrons. This view is strengthened by the existence in each case of an alkali metal cation of similar structure: perhaps quite as remarkable as the electrides, these contain one or two alkali metal anions in each cavity⁵.

Singh *et al.*¹ tackle the electride $Cs^+(15\text{-crown-5})_2 e^-$, whose optical, electrical and magnetic properties indicate that the electrons are individually trapped at separate sites and have only weak exchange interactions with one another^{3,6}. But electrons are not like ordinary anions — their small mass leads to pronounced quantum effects such as 'leakage' onto surrounding nuclei some distance away and antiferromagnetic transitions due to electron–electron interactions.

Their calculation is a *tour de force* which uses the known crystal structure and a large collection of plane waves and atomic orbitals to compute electron densities and energy bands. Although the method used (local density approximation) does not yield correct energies, it has provided reliable electron density distributions and other ground-state properties, so we can probably regard the general conclusions as trustworthy. A previous band-structure calculation⁷ also concluded that the electron distribution is centred at the anionic site, but, according to Singh *et al.*, for the wrong reason.

One usually assumes that electrons seek a potential energy minimum. If Singh and colleagues are correct, electrides are an exception. Interaction with the 'core' electrons of the cation and the complexant electrons, and the availability of a 'roomy' site at the vacancy, combine to trap the electron in the region of a potential maximum. The total energy is minimized by spreading the electron density throughout the large cavity, thereby reducing its kinetic energy. Such descriptions are admittedly an attempt to fit the multi-electron reality into a one-electron picture. Nevertheless, electrides may provide unusual examples of electron trapping that results from the tendency to minimize the kinetic energy by occupying a large empty space despite the existence of a maximum in the potential energy in that region.

This theoretical treatment may help to

explain a recent puzzling experimental finding⁸ for the similar electride, $Cs^+(18\text{-crown-6})_2 e^-$. Earlier magnetic susceptibility measurements had indicated the presence of very weak electron–electron interactions with energies equivalent to thermal energies at only a few kelvin. But the new measurements on crushed crystals that had always been kept cold showed much stronger magnetic coupling, equivalent to thermal energies at 30 to 50 kelvin. When warmed above 230 kelvin and re-cooled, the samples reverted to weak magnetic coupling. How was the magnetic coupling destroyed?

The new theory¹ shows that the electron density distribution is highly anisotropic, with higher density along the narrow channels, directed along the b -axis, that connect the trapping sites than in other

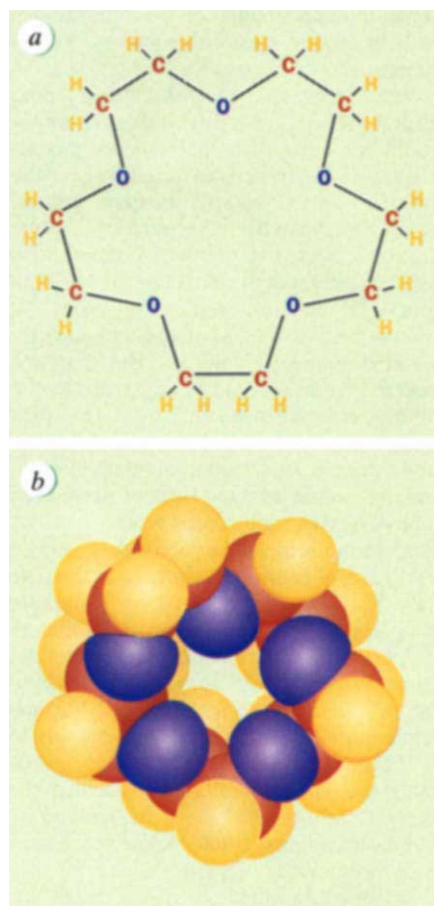


FIG. 1 a, Structural formula and b, space-filling representation of 15-crown-5, the complexant in the electride studied by Singh and colleagues. Two such molecules tend to form a 'sandwich' about a caesium cation because of the attraction of the positive charge of Cs^+ to the partial negative charges on the oxygen atoms.

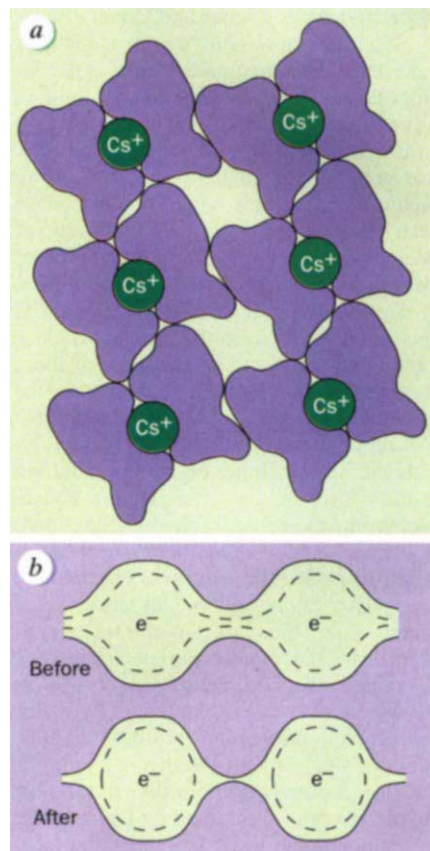


FIG. 2 a, Schematic diagram of the packing in $Cs^+(15\text{-crown-5})_2 e^-$. This is a view down the b -axis, and shows the snug fit of the large complexed cations to one another and the presence of empty channels that connect electron trapping sites (the cavities). Each cavity is surrounded by eight complexed cations. b, How the theory of Singh *et al.* might account for the decrease in electron–electron coupling seen when an electride becomes disordered as the temperature is raised⁸. If the narrow channels through which the electrons communicate are blocked by the disordered crown ether molecules, the coupling will decrease. The disordering is reversible in $Cs^+(15\text{-crown-5})_2 e^-$ but not in $Cs^+(18\text{-crown-6})_2 e^-$.

directions. Electron–electron overlap in adjacent sites is helped by the presence of open channels. (Figure 2a shows the packing and channels in this electride.) Thermal disorder in Cs^+ (18-crown-6) $_2\text{e}^-$ may block the similar narrow channel that also exists in this electride, causing the electron density to ‘withdraw’ into the cavity and decreasing the electron–electron overlap (Fig. 2b). During the discussion at a 1969 conference on electrons in fluids, the late Professor Lars Onsager likened the solvated electron in ammonia to an amoeba, a handy metaphor here: the high polarization allows distortion of the electron distribution to fill empty spaces as much as possible. When the channels are closed off, the ‘amoeba’ pulls back into the cavity and becomes more nearly spherical.

Singh and colleagues are not alone in wrestling with the nature of electrides. Another theoretical approach has been developed by Rencsok *et al.*^{9,10}, who considered a model electride ‘molecule’ of $\text{Li}(9\text{-crown-3})_2$ in the gas phase. From extensive quantum calculations for this molecule, they concluded that the electron occupies a Rydberg-like state, with most of the electron density well outside the complexed lithium cation — in effect a large pseudo-atom with a single electron outside a large positive core. To approach the solid state, they aim to use this high-quality molecular wavefunction as the basis for cluster and solid-state calculations.

Electrides form a fascinating class of crystalline materials in which the ‘excess’ electrons do not ‘belong’ to any particular atom or molecule. Our picture of them is already becoming clearer: vacant trapping sites, narrow channels and the large separations of these sites lead to electron localization. Theoretical studies like these should improve our understanding of the diverse properties of the known electrides and guide us in the synthesis of others. □

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When order is disordered

Bruce J. Ackerson

ENTROPY can give unexpected results. That is the message of a paper on page 35 of this issue¹, where Eldridge, Madden and Frenkel present computer simulation results for a mixture of two different sizes (A and B) of hard spheres subject to thermal agitation. Remarkably, this simple model system exhibits a rich variety of crystalline structures, including a superlattice order having a regular 112-particle repeat unit containing 13 B particles for every A particle.

At first glance the existence of complex crystalline structures may not seem surprising, because we are surrounded by highly organized states of matter. These states of matter (such as you the reader) are usually not at equilibrium and rely on a flow of matter and energy for continued existence.

But if the system under study is sealed in a box that prevents the flow of matter and energy, equilibrium eventually results. Equilibrium is a state of maximum entropy, commonly referred to as a state of increased or maximum disorder. For example, an open bottle of perfume in a sealed box eventually fills the enclosure with its fragrance. The dispersion of the perfume molecules represents a state of increased disorder compared to the initial state. We would be surprised if a second empty bottle placed in the box decreased the disorder by spontaneously collecting all the perfume molecules in it.

On the other hand, we are familiar with supersaturated sugar solutions which form sugar crystals. The principle that entropy is maximized for the whole system of sugar solution plus crystals leads to a minimum free energy principle² for the subsystem containing only the crystals. The final crystal entropy (and disorder) is lower than that for the initially dispersed sugar molecules, but the ordering is driven by the attraction of the sugar molecules for one another. This attraction leads to a reduced energy for the sugar molecules in the crystal and has a greater influence on reducing the free energy than does the entropy change in the subsystem.

What if the molecules or particles in the system are more like microscopic billiard balls? That is to say, they only repel one another at contact. Will such a system spontaneously organize into a crystal structure if the particles occupy roughly half of the available volume? Under these conditions there is no force ‘holding’ the particles in a crystalline lattice order. There is only the random thermal motion of the particles which leads to maximum entropy (disorder) in equilibrium.

George Uhlenbeck posed a similar

question in 1957 to the luminaries of equilibrium physics — the mentors of the current generation of statistical physicists³. He had asked, he said, at an earlier round table discussion in Seattle whether an order–disorder transition exists for hard spheres. There, the votes for and against had been even; what did his present audience believe would happen? With sixteen members voting the result was — even again!

How far have we come since then? Computer simulations⁴ of molecular dynamics have indicated with increasing persuasiveness that a transition to a crystalline state would occur when the occupied space is more than about half the available space. Furthermore, it is now possible to manufacture spheres⁵ less than a micrometre in diameter, thought to behave like microscopic billiard balls when suspended in a solvent. The spontaneous organization of these particles into crystalline order^{6,7} corroborates the computer-simulation results.

But this seems contradictory to the intuitive notion that increased entropy means increased disorder. Must our intuition be corrected?

Let us assume that the solvent plays a neutral role and concentrate on the suspended particle order at local and global scales. To this point we have been concentrating on the global picture, where the organized crystalline structure has a lower entropy than the disorganized random or liquid structure (a normal intuitive association of entropy with disorder). Locally, however, there is more jamming together in the random structure than in the crystalline structure. Thus, there are more ways or possibilities for moving the particles around a little bit in the crystalline structure than in the random structure. So there is a higher local entropy in the crystalline state than in the random structure, and, reassuringly, increased entropy is again associated with increased disorder. The transition to a crystalline structure occurs when the local entropy gain outweighs the global entropy loss of the crystalline state

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relative to the random structure.

Further layers of subtlety emerged when experiments^{8,9} showed that mixtures of different sizes of submicrometre spheres could lead to unusual superlattice structures in equilibrium. There was the nagging doubt, though, that the superlattices arose not from entropy but from some experimental effect that had been neglected. Perhaps there were residual distance-dependent forces between the

spheres. The calculations of Eldridge and co-workers set these doubts to rest: these superlattice structures can indeed result from purely entropic effects. To me, their work demonstrates the subtle nature of disorder in systems that 'appear' highly ordered. □

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PHARMACOLOGY

Medical uses of marijuana?

Leslie L. Iversen

MARIJUANA, and its active ingredient Δ^9 -tetrahydrocannabinol (Δ^9 -THC), have been used in medicine for many centuries. Its diverse uses include the treatment of pain and inflammation, the lowering of intraocular pressure in glaucoma, the relief of nausea associated with cancer chemotherapy, and the relief of muscle spasms associated with multiple sclerosis¹. Some of these applications are better validated than others, and some have been superseded by the advent of other powerful medicines, as in the treatment of glaucoma by β -adrenoceptor blockers or pilocarpine, and the treatment of the sickness associated with cancer chemotherapy by antagonists to the 5-hydroxytryptamine receptor 5-HT₃. The exploitation of the possible medical benefits of marijuana, however, has always been limited because of its powerful psychoactive properties, and there is currently no

approved medicinal use in the United Kingdom.

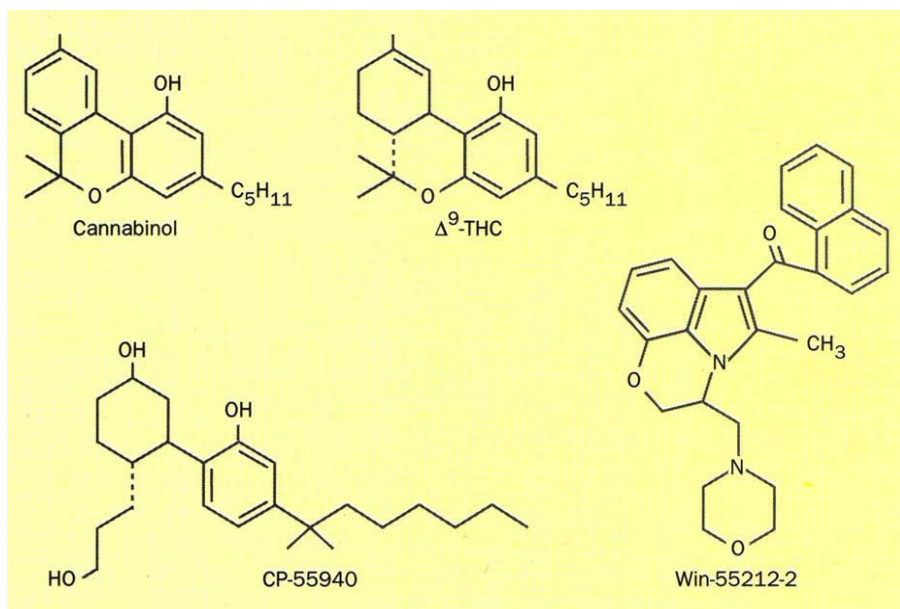
The discovery of a new type of cannabinoid receptor, reported by Munro *et al.* on page 61 of this issue², thus offers new horizons for the potential use of cannabinoids in medicine, for this receptor is not present in brain but only in peripheral tissues. Using polymerase chain reaction techniques Munro *et al.* identified six classes of clones expressed in a human leukaemic cell line with homology to G protein-coupled receptors. Some of these appeared to correspond to known G protein-coupled receptors but others were novel. One of these, termed 'CX5', showed some homology with the cannabinoid receptor, whose identification and cloning from rat brain was described three years ago by Matsuda *et al.*³. When the CX5 complementary DNA was transfected into tissue culture cells and expressed,

its identity as a cannabinoid receptor gene was confirmed by the high-affinity binding of the synthetic high-affinity cannabinoid receptor radioligands CP-55940 and Win-55212-2. Furthermore, Δ^9 -THC displaced these radioligands with high affinity, whereas the biologically inactive analogue cannabidiol was much less effective. Further experiments revealed that expression of messenger RNA for the CX5 gene could only be detected in rat spleen, and not in rat brain, liver, nasal membranes, thymus, lung or kidney. Within the spleen, *in situ* hybridization experiments revealed the presence of high densities of CX5 in the marginal zones around the periarteriolar lymphoid sheaths.

Many questions remain unanswered. Is the second cannabinoid receptor expressed only in the spleen? The high level of expression, probably in the highly active macrophages, in a region where the outside world meets the immune system, suggests a possible role in inflammatory and immune responses to infection or other foreign antigens. Might CX5 be found more widely in macrophages elsewhere? The inducibility of CX5 by dimethylformamide reported by the authors in the human cell line may suggest that macrophage expression of CX5 will prove similarly inducible. Among other tissues that might be examined, the testis is important as this is the only peripheral tissue that exhibits some expression of the brain-type cannabinoid receptor. An issue raised by the therapeutic use of cannabinoids has been their effects on male fertility and ability to lower testosterone levels⁴.

What is the endogenous ligand for the CX5 receptor? The authors report that the lipid derivative anandamide — described recently as a possible endogenous ligand for the brain receptor⁵ — had some ability to displace radioligands from the CX5 receptor, but with an apparent affinity some thirty times less than for the brain receptor. The close association of CX5 sites with macrophages may suggest some other product of arachidonic acid metabolism as a possible candidate.

Whatever the answer to these questions, there is little doubt that the newly discovered peripheral cannabinoid receptor will help us to understand better the complex pharmacology of this diverse group of chemicals. The feasibility of designing drugs that exhibit selectivity for the peripheral versus brain receptor is already indicated by the observation that the Δ^9 -THC analogue cannabidiol showed some selectivity for the CX5 site com-



Cannabinoid structures. Δ^9 -THC is the psychoactive ingredient of marijuana. The closely related analogue cannabidiol is considerably less psychoactive than Δ^9 -THC, but is similar in affinity to Δ^9 -THC at the newly discovered CX5 sites. The synthetic compounds CP-55940 and Win-55212-2 are more potent than Δ^9 -THC and more water soluble; they have proved valuable tools as selective radioligands for cannabinoid receptors.

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pared with the brain receptor. Further medicinal chemistry efforts could no doubt yield highly selective ligands. It is possible that such compounds might represent novel anti-inflammatory or immunosuppressant agents. The discovery by Munro *et al.* highlights yet again the ability of molecular biology to discover

new receptors at a rate much faster than pharmacologists can handle. □

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HIGH-ENERGY ASTROPHYSICS

Twice is not enough

Kevin Hurley

THE heavens are anything but immutable at high energies. Sources of X-rays and gamma-rays come and go on all timescales. The most fleeting high-energy phenomena are the X-ray bursters, the soft gamma repeaters and the gamma-ray bursters. The X-ray bursters are fairly well understood in terms of thermonuclear explosions and accretion onto neutron stars, the dense remnants of massive but otherwise normal stars which have finished their lives by exploding as supernovae. In contrast, little is known about the soft gamma repeaters or the gamma-ray bursters, largely because until now no one has been able to find a completely convincing quiescent counterpart to either in any part of the electromagnetic spectrum. Now Kulkarni and Frail, on page 33 of this issue, have announced the discovery of a supernova remnant whose location is consistent with that of a soft gamma repeater.

Observationally, the three classes of high-energy transients are easy to distinguish. The three dozen or so known X-ray bursters have soft spectra, typical of blackbodies with temperatures of about 1 keV coming from a surface area of a few square kilometres, and the sources are observed to burst repeatedly over timescales of years. Only three soft gamma repeaters are known, and their energy spectra may be characterized by that of a hot gas (thermal bremsstrahlung) at a temperature of 40 keV. One, SGR1806–20, has been observed to burst well over 100 times. Another, the source of the bright anomalous 1979 March 5 event, has burst 17 times. The third, B1900+14, burst three times in three days in 1979 and three times in two months in 1992. Finally, the gamma-ray bursters have hard energy spectra, which have been measured to over 100 MeV. More than a thousand have now been observed, and there is no compelling evidence that any single source has burst more than once.

There has been no consensus on the

origin of either the soft gamma repeaters or the gamma-ray bursters. Indeed, various theories have placed them at distances of a few hundred parsecs or throughout the depths of the Universe. A tantalizing clue in this puzzle was that the location of the 1979 March 5 source is consistent with a supernova remnant, N49. However, because it is in the Large Magellanic Cloud at a distance of 55 kpc,



The N49 supernova remnant in the Large Magellanic Cloud. This remnant probably contains a neutron star which generated the very intense 1979 March 5 burst, and 16 subsequent weaker events. The radio supernova remnant reported by Kulkarni and Frail lies in a highly obscured region, towards the Galactic Centre, and cannot be detected at optical wavelengths.

an exceptionally large luminosity, 5×10^{44} erg s^{-1} , is implied. Thus theorists have been led to sources either more mundane (nearby neutron stars) or more exotic (multiple, gravitationally lensed images of a single explosion at cosmological distances). Still, the apparent directions of the two other soft gamma repeaters — near the Galactic Centre for SGR1806–20, and close to the galactic plane for B1900+14 — hinted at a unified explanation for all three sources as neut-

ron stars in supernova remnants.

Kulkarni and Frail's announcement that the position of the galactic supernova remnant G10.0–0.3 is consistent with that of SGR1806–20 supports this view. So we now have two identifications of soft gamma repeaters that point to a neutron star origin in a young (less than 10,000 years old) supernova remnant, and many, many ideas about what may cause them to burst, from accretion to starquakes. But there are probably several hundred such remnants in the Galaxy, and only three soft gamma repeaters, even though the soft gamma repeaters seem to be bright enough to be detected by current instruments whatever their distance, as long as it is galactic. Kulkarni and Frail speculate that only neutron stars with certain properties — magnetic field strength and rotation rate in a certain range, for example — evolve to become soft gamma repeaters. And they may not remain in this phase for very long, so it could well be that three or so is indeed the total number that are currently active.

What of the third soft gamma repeater, B1900+14? Its location is nowhere near as precisely known as the other two, and there are no known supernova remnants precisely in its error box. But if guilt by association can be accepted as evidence, then this too can plausibly be assumed to be related to a neutron star in a young, galactic supernova remnant. Proof may be forthcoming if current searches bear fruit. This may well lay to rest the cosmological theories of the origin of the soft gamma repeaters.

And the ultimate mystery, the gamma-ray bursters? The genealogy of high-energy transient sources is too poorly known to conclude that soft gamma repeaters and gamma-ray bursters are related, although there have been theories to this effect. For one thing, even if every neutron star in the Galaxy were a gamma-ray burster, there would still not be enough to have each one burst only once. On some timescale, perhaps hundreds of thousands of years or more, they would have to repeat. For another, the light curves and energy spectra of the two classes are vastly different.

Although such objections can always be overcome by evoking source evolution, there is still no hard evidence to link them. So for the time being, the identification of two out of three soft gamma repeaters appears to solve only the second of three mysteries surrounding the origin of high-energy, short-timescale transients. □

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Question of commitment

Jim Manley

INTRONS, or non-coding sequences, are removed from messenger RNA precursors in a complex splicing process. Not only is the reaction pathway complicated, involving such baroque features as RNA lariats and branchpoints, but the number of factors required to catalyse the reaction is enormous, involving multiple small nuclear ribonucleoprotein particles (snRNPs) and an unknown (but large) number of accessory proteins. Despite this complexity, there has been considerable progress over the past decade in understanding the details of the reaction. But many crucial questions remain, one of which concerns the nature of the very first steps in the splicing reaction. How is a particular pre-mRNA initially recognized and committed to splicing and how are intron boundaries (splice sites) identified? This issue is especially important in multicellular organisms, where most mRNA precursors contain numerous introns, and many are subject to complicated patterns of alternative splicing. On page 82 of this issue¹, Xiang-Dong Fu provides part of the answer to the commitment question, and it turns out to be surprisingly simple. It seems that the presence of a single protein, one of the family of SR proteins, is sufficient to enable the splicing machinery to select a particular RNA in an *in vitro* splicing reaction.

Fu finds that, in an *in vitro* system, one SR protein — SC35 — is sufficient to allow human β -globin pre-mRNA to be preferentially spliced when an excess of a competitor RNA and splicing extract were added subsequently (the operative definition of commitment), whereas another human SR protein — ASF/SF2 — cannot. With an HIV tat RNA splicing substrate, the situation was reversed.

These findings are surprising in the light of earlier studies, carried out first in yeast^{2,3} and then in mammalian^{4,5} systems, which suggested that the U1 snRNP particle has a key early role in commitment of a pre-mRNA to splicing. It is well established that U1 snRNP recognizes the 5' splice site of the intron, but it also appears that this interaction by itself is relatively weak, suggesting the need for an additional factor(s). Indeed, the experiments showing a requirement for U1 snRNP in the formation of the earliest detectable splicing complexes by necessity used unfractionated cellular extracts and relied on the ability to separate and identify snRNP-containing complexes. It is certainly possible that a simple protein-RNA complex could have gone undetected.

SR proteins enter the picture from a somewhat different perspective. They

constitute a group of at least six proteins that are highly conserved from the nematode *Caenorhabditis elegans* to humans⁶. (They seem not to exist in yeast, however, perhaps indicating a mechanistic difference in an early step in splicing.) The prototypical member of this family is the human protein ASF/SF2, which was identified independently by two distinct *in vitro* assays. In one⁷, it was purified as an activity able to switch the utilization of two 5' splice sites in an alternatively spliced pre-mRNA, thereby recapitulating *in vitro* a cell-specific pattern of splicing observed *in vivo*. In another⁸, the protein was purified by its ability to activate splicing of a simple pre-mRNA in a depleted extract, indicating that ASF/SF2 can function as an essential, as well as an alternative, splicing factor.

The sequence of the protein revealed interesting features^{9,10}, which indeed seem to be common to all SR proteins, including the well studied essential splicing factor, SC35 (ref. 11). At the amino terminus is an RNP-type RNA-binding domain (or RNA-recognition motif), undoubtedly involved in binding the pre-mRNA, and at the carboxy terminus is a region (called the RS domain) that consists of repeating arginine and serine residues (hence the name SR proteins). These features are intriguing because they are reminiscent of genetically defined regulators of splicing in *Drosophila*¹². RS domains have now been found in a number of splicing factors, and, indeed, the presence of such a region can probably be considered diagnostic of a protein involved in splicing. Although the precise function of RS domains remains to be established, an attractive model, for which there is already some support¹³, is that they constitute activating regions, perhaps analogous to the acidic domains found in certain transcription factors. Indeed, it may be that splicing and transcription activators share a similar modular organization, each containing a domain that binds nucleic acid and a discrete activating region.

Initial work suggested that SR proteins might be functionally interchangeable^{6,14}, raising the possibility that their activities could be redundant *in vivo*. However, several recent studies, including the present work by Fu, detect differences in the behaviour of certain SR proteins. A *Drosophila* SR protein (Rbpl) and ASF/SF2 display significant differences in their ability to function as essential as well as alternative splicing factors¹⁵, and several mammalian SR proteins also differ from each other in their effects on alternative splicing¹⁶. The activation of the female-

specific intron in the *Drosophila double-sex* pre-mRNA by Transformer and Transformer-2 proteins also requires SR proteins, and this requirement can be met only by a subset of these proteins¹⁷. So the different SR proteins may have distinct roles *in vivo*, perhaps modulating the splicing of specific classes of transcripts. The idea that SR proteins may be involved in regulation is also supported by differences in their tissue distribution¹⁶, the existence of alternatively spliced forms⁷, and by the ability of an snRNP-associated protein kinase to phosphorylate serines in the ASF/SF2 RS domain¹⁸.

The findings of Fu¹ are important not only because they suggest an early determinative role for SR proteins in the assembly of splicing complexes on pre-mRNA, but also because they add to the view that SR proteins perform distinct functions that may be involved in splicing control. The simplest explanation for the differential action of SC35 and ASF/SF2 is that the two proteins, and, by extension, other SR proteins, have distinct sequence preferences in their interaction with pre-mRNA.

Like many unexpected findings, Fu's results raise at least as many questions as they answer. For example, which RNA sequences are recognized by SR proteins, and do different proteins recognize different sequences? What do the proteins do after binding to the pre-mRNA? And do all SR proteins do the same thing? The answers to these questions will undoubtedly provide exciting insights into the mechanism and control of metazoan pre-mRNA splicing, but the important role of SR proteins in the earliest steps of the process now seems firmly established. □

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Towards healthier infertility

Olivia P. Judson

SINCE the invention of the combined oral contraceptive pill in 1951, most chemical contraceptives have been refinements (such as lower dosages) or variations (such as method of delivery) on the same oestrogen-progestogen theme. Radical shifts in approach have not happened¹. This may at last be changing. Writing in *Contraception*, Spicer and colleagues² report the results of a two-year pilot trial of a new contraceptive that is not only different in hormonal make-up, but is also one that they believe will reduce the relative risk of breast cancer for women who use it.

Here's why they think it will work. In evolutionary terms, modern Western women are leading a strange new life. Even as recently as two hundred years ago, the average number of menstrual cycles a woman went through in a lifetime was as low as 30. Today, that number for a woman with two children is closer to 450. This enormous increase in the number of menstrual cycles is due to several factors, including earlier age at menarche (now 12 or 13 rather than 17 or 18), bearing fewer

children, and breast feeding those children for a shorter period of time³. A monthly menstrual cycle means a monthly exposure to high, fluctuating levels of the steroid hormones oestrogen and progesterone, hormones that promote cell proliferation; cell proliferation is a risk factor for cancer⁴.

Every menstrual cycle starts with the release of a peptide hormone, the follicle-stimulating hormone, from the pituitary. This hormone stimulates the development within the ovaries of several follicles, each of which contains an immature egg. After a few days (usually around day six of the cycle) one follicle becomes dominant and begins to grow quickly, and at the same time secretes high levels of oestrogen into the blood stream. Oestrogen triggers proliferation of endometrial cells, replacing the cells lost at menstruation and rebuilding the endometrial lining in preparation for implantation of a fertilized egg. As the follicle matures, oestrogen secretion reaches a peak. This triggers the release of a second pituitary peptide hormone,

luteinizing hormone, which in turn triggers ovulation. At ovulation, the follicle ruptures the surface of the ovary and releases the egg. The follicle then fills with a blood clot which is soon replaced by proliferating cells; this whole structure is known as the corpus luteum. The corpus luteum secretes both oestrogen and progesterone into the blood stream. The presence of progesterone prevents further proliferation of the endometrial lining. But, oestrogen and progesterone together stimulate mitotic divisions in the breast⁴.

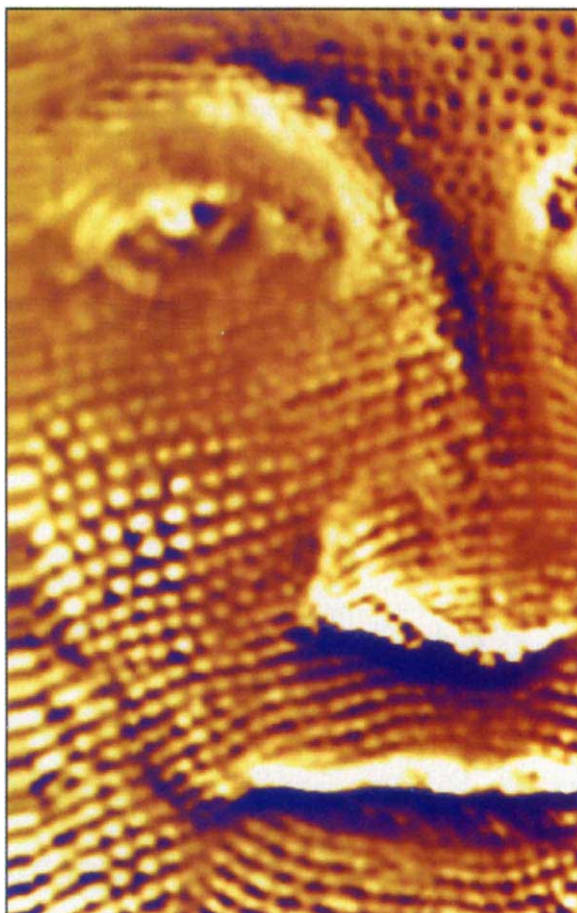
If implantation does not occur, the corpus luteum regresses, concentrations of oestrogen and progesterone drop to their lowest levels, the uterine capillaries rupture, menstruation takes place, and the cycle repeats. Thus, just as frequent sunbathing increases the risk of skin cancer, regular exposure of breast, ovarian and endometrial tissue to high levels of hormones increases the risk of cancers of the breast, ovary and endometrium. As this argument predicts, the relative risk of all three cancers increases much more slowly after menopause, or hysterectomy, than before⁵.

The original, high-dose pills, made of synthetic oestrogens and progestogens, have been shown to reduce the relative

Magnetic personality

GREEK god or Inca mask? No, this is the impression of George Washington on the US dollar bill, imaged by magnetic microscopy. The basis of this microscope is a high-transition-temperature SQUID (superconducting quantum interference device), made by laser deposition of a thin film of YBCO ($\text{YBa}_2\text{Cu}_3\text{O}_{7-x}$) on SrTiO_3 . By raster-scanning the SQUID across the note, a picture is built up from variations in the magnetic field associated with individual patches of the ferro-magnetic ink that make up the president's features. As a guide to those who do not have a reference source in their wallets, Washington's nose is about 2 mm across.

Magnetic images are not purely decorative. With a sufficiently sensitive detector, the tiny magnetic fields produced by current in a circuit can be mapped, or isolated regions of superconducting material detected by their characteristic expulsion of magnetic flux. The particular device responsible for this image is the brainchild of R. C. Black and co-workers (*Appl. Phys. Lett.* 62, 2128–2130;



1993) together with J. R. Anderson, A. Thanawalla and A. Piqué. It has unusually good field resolution — the false colours in this picture denote fields from +320 nT (white) to -320 nT (deep purple), with a resolution of about 200 pT. The spatial resolution is about 80 μm , good enough for imaging currents in fine wires or patterns etched by photolithography. As the high- T_c SQUID operates at 77 K, it should be cheap to run: conventional low- T_c SQUIDs must be cooled by liquid helium, which is more expensive than liquid nitrogen. The usual method is to put just the SQUID and its pick-up coil into a dewar of helium; the thickness of the dewar wall, and not the size of SQUID, is then the chief limit on spatial resolution. But here the authors immerse both sample and detector in nitrogen and adjust the separation to 60–200 μm . If the aim is to scan microelectronic circuits in operation, then liquid nitrogen again has the edge. In liquid helium, however sensitive the SQUID, little action will be seen; the silicon components will not run at such low temperatures. Lindsay Matthews

risk of ovarian and endometrial cancers^{6,7}. They are believed to do this by preventing cell proliferation. In the ovaries, this means blocking development of follicles and preventing ovulation, and in the endometrium, this means blocking the build-up of the endometrial lining that would usually take place in the presence of unopposed oestrogen. However, the relative risk of breast cancer has not been improved by use of the pill, probably because mitotic divisions in the breast are at their highest rate under the influence of oestrogen and progesterone together⁴.

Spicer and colleagues have returned to an early idea for contraception — abandoned in the 1970s because it did not seem to have any advantages over the existing methods — which is based on a gonadotropin-releasing hormone agonist (GnRHA). Gonadotropin-releasing hormone controls all the hormones involved in the female reproductive system. Synthesized in the hypothalamus, it is usually released by the brain in pulses and stimulates the release of follicle-stimulating hormone and luteinizing hormone. However, given on its own at a constant level, this hormone shuts down all reproductive function, essentially inducing menopause. Constant administration of GnRHA has the same effect. To prevent the bad aspects of menopause, such as hot flushes and bone loss, the researchers add back a small dose of oestrogen, and, to prevent hypertrophy of the endometrial lining, a small dose of progesterone is given once every four months to induce menstruation. Thus menstrual periods would happen only three times a year and the doses of oestrogen and progesterone would be lower than those found in Western women, even at the start of their cycle; freedom from hormonal fluctuations should also eliminate other side effects of continual cycling, such as premenstrual syndrome².

Although it would take decades to be certain that such a scheme reduces cancer risks and does not create any other health hazards⁸, the results of the trials so far are promising. Women taking the contraceptive seem to show reduced mitotic activity in the breast, and show no premenstrual syndrome. Initially the subjects were losing bone density, but the addition to the regimen of a small amount of an androgen, which would otherwise be produced by the ovaries, seems to have prevented further loss. As an added

benefit, contraceptive subjects showed an increase in high-density lipoprotein cholesterol. At the moment, as the regimen relies on compounds that are already available for prescription, it is too complicated to be practical. It involves an injection of GnRHA once a month, a low-dose oestrogen pill six days out of seven, and a progesterone pill for thirteen days once every four months. The ultimate goal is delivery of all compounds together².

At present, cancers of the reproductive

tract are largely a Western problem. But as the lifestyles of women in non-Western countries change with improved nutrition and fewer children, the relative risk of these cancers to non-Western women will increase accordingly. A path towards healthy infertility will be welcome worldwide. □

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CONSERVATION BIOLOGY

Cougars and corridors

Jared Diamond

As large expanses of natural habitat become fragmented by human use, large populations of plants and animals become fragmented into sets of small populations, each of which may be too small to survive in isolation. To counteract this process, conservationists have recommended reconnecting the fragments into an effectively larger expanse by habitat corridors. But this strategy is controversial: how much do corridors enhance population viability, do animals really use them and might they do more harm than good? Paul Beier has now addressed these questions for a population of cougars by combining mathematical modelling with radiotelemetry studies (*Conserv. Biol.* 7, 94–108; 1993).

The cougar (alias puma or American mountain lion) is the last widespread species of top predator in the United States, although it has become extinct in much of its range and endangered in other parts. Because it lives at low densities (one adult per 100 km²) and gives birth at long

intervals (two years), it is an excellent indicator of ecosystem health: where cougars survive, so can more abundant species. Cougar populations have a skewed sex ratio, with about two adult females for each male. Hence dispersal, especially of juvenile males, might be critical to sustaining cougar populations.

Beier's study area was the Santa Ana Mountains near Los Angeles, scene of the familiar horror photos of housing developments viewed through a pall of smog behind freeways. Coexisting with more than a million people are about 20 adult cougars, now restricted to 2,070 km² of natural habitat, an island in a sea of houses and golf courses. Unfortunately, that island is becoming dissected by freeways and developments, and only 1,114 km² of the 2,070 km² forms a contiguous piece of protected habitat.

Beier simulated the fates of hypothetical cougar populations by incorporating cougar demographic parameters into mathematical models. He defined a viable

Paul Beier

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Incompatible habitats — people and cougars live cheek by jowl in southern California.

population as one with a 98 per cent or more probability of surviving for a century. By that criterion, 2,200 km² of habitat were required to support a viable cougar population without any immigration. As habitat area decreased, risk of extinction increased, usually because of the death of the last breeding male at a time when there was no surviving male cub. So viability was greatly improved by male immigration — immigration of only one male per decade decreased the minimum critical area necessary for population viability by hundreds of square kilometres.

How realistic are Beier's simulations? One indication is that a 75-km² area of cougar habitat in the western Santa Anas, isolated by urban development in the late 1970s, had lost its cougars by 1990. Another is the reproductive failure observed by Beier and colleagues in 1988, a failure apparently triggered by the death, in February 1988, of the territorial male in the southern half of the range. Thereafter, no sign of an adult male — nor of breeding by seven radio-tagged and about four untagged females — was evident for more than a year. Finally, after two young males reached breeding age in early 1989, copulatory noises were heard in April 1989 from four of the marked females, three of which gave birth to cubs three months later (the gestation period in cougars). By the summer all but one of the marked females had cubs. This temporary loss of breeding males and consequent threat to the population are as predicted by Beier's simulations.

In the Santa Anas, the barriers to cougar dispersal are urban development and freeways, while the habitat corridors include obstacles and constrictions, most notably underpasses and culverts under freeways. Do cougars use these corridors? Radiotelemetry showed that they do, and that cougars ignoring the underpasses do so at their peril. One telemetered individual was tracked coming up to a freeway, waiting there for hours while apparently trying to get up its courage, then making a dash into the road — and immediately being struck by a vehicle and killed.

The sole remaining potential source of cougar immigration to the Santa Anas is the Palomar Range to the east, connected by the tenuous Pechanga corridor 4.5 km long and as little as 50 m wide along a creek. Adjacent night lighting, golf courses, concrete embankments and other corridor features unattractive to cougars culminate in the I-15 freeway, which can be safely crossed only by a single underpass at a bridged river. It seems that cougars have difficulties finding or traversing the Pechanga corridor, as three were killed on I-15 south of the underpass, another ended up in a residential area and was captured, and a fifth followed the

corridor in the wrong direction (that is, emigrated from the Santa Anas), ran straight across I-15 and was lucky to survive.

At the June 1993 meeting of the Society for Conservation Biology, Beier summarized data on nine dispersing juveniles and three of the main corridors. Each corridor was used by two or three radio-tagged dispersers, and five of the nine juveniles dispersed through corridors; two especially aggressive explorers each used two corridors. In addition, four dispersers explored narrow peninsulas of habitat extending into urban areas: if those peninsulas had led to large expanses of habitat blocks, then eight of the nine juveniles would have successfully dispersed via corridors. Clearly, cougars are adept at finding and using corridors — if they are preserved.

Does all this mean that the future of the Santa Ana cougar population is secure? The answer is 'no'. Beier's calculations address its demographic viability but not its genetic viability. Big cats are notorious for their susceptibility to reduced fertility from inbreeding, and the 20 cougars of the

Santa Anas will surely need fresh cougars to be brought in by humans if they are to escape inbreeding depression. In addition, the Santa Anas are a land-use nightmare, being subject to five county governments, 17 municipalities, two transportation agencies and the world's largest and politically most powerful water district. An unfavourable decision by any one of these agencies could effectively bisect the local cougar population.

The larger message is that corridors for wildlife can indeed be vitally important in conservation, and that telemetry is a good tool to determine whether they are used. Current methods of identifying corridors are either to look at an aerial photo and guess where one should be, or else to proclaim that a bridge or a left-over scrap of habitat fills that function. Beier's study demonstrates the virtues of letting animals themselves instruct us about corridors and their value. □

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TUMOUR SUPPRESSOR GENES

No room at the p53 inn

Jennifer A. Pieterpol and Bert Vogelstein

IDENTIFYING proteins that bind to the transcription factor and tumour suppressor protein p53 has become a major focus of research, the idea being that such associated proteins will provide clues to p53 function. As a result, the p53 inn is rapidly filling with guests, including several viral and cellular proteins. Three recent articles¹⁻³, including one on page 79 of this issue by Dutta *et al.*, reveal yet another protein that has checked in, replication protein A (RPA).

RPA is a multisubunit complex containing three polypeptides of M_r 70,000, 34,000 and 13,000. The binding of RPA to single-stranded DNA may be the initial step in DNA replication, and seems to be

required for unwinding DNA origins. The importance of RPA activity is underscored by the discovery that all three genes for the three subunits of RPA are essential for yeast viability⁴. A role for RPA in human DNA excision repair *in vitro*⁵ has also been demonstrated.

Dutta and colleagues³ show that full-length p53 interacts with and inhibits the ability of RPA to bind single-stranded DNA *in vitro*. In the context of growth control, this interaction could regulate the onset of the S phase of the cell cycle by directly inhibiting the ability of RPA to bind to replication origins. This finding is provocative because it may represent a molecular basis for the previously observed arrest of cells in the G1 phase which is mediated by increased levels of p53. However, mutant forms of p53, which are unable to induce G1 cell-cycle arrest, also bind RPA and inhibit its binding to single-stranded DNA. The authors recognize this and are cautious in the interpretation of their data.

Li and Botchan¹ and He and colleagues² report that the acidic activation domains of several transcription factors, including p53, are able to bind selectively to human and yeast RPA *in vitro*. These acidic domains, when fused to the Gal4 DNA-binding domain, can activate replication as well as transcription from replication origins if such origins contain a Gal4

Table 1 p53-associated proteins

Cellular proteins

CBF, CCAAT binding factor
E6-AP, mediates HPV E6 interaction with p53
HSP70, heat shock protein 70
MDM2, cellular oncoprotein
RPA, replication protein A
SPI, general transcription factor
TBP, TATA binding protein
WT1, Wilms tumour gene product

Viral oncoproteins

Ad5 E1B, adenovirus type 5 E1B
EBNA-5, Epstein-Barr nuclear antigen 5
HPV16/18 E6, human papilloma virus 16/18 E6
SV40 Tag, simian virus 40 large T antigen

operon. These studies accentuate the intriguing relationship between transcription and replication; the new work links the tumorigenesis-associated protein p53 with these ubiquitous cellular processes.

As increasingly sophisticated techniques to identify associated proteins become available, there will probably be an escalation in the number of proteins that can be shown to bind p53. But determining which associations are physiologically relevant for p53 function is not simple. It is unlikely that p53 can bind to all the cellular proteins listed in Table 1 at the same time. Clearly, either cell-type or cell-cycle specificity must be invoked to accommodate all these guests. It is essential to determine what percentage of cellular p53 is bound by each associated protein and how the portion that is bound affects p53 function.

This raises several unanswered questions about p53, concerning its true cellular function(s). Over the past several years a number of biological and biochemical activities have been ascribed to p53 (ref. 6 and Table 2). The existence of mature p53 'knock-out' mice suggests that p53 is not necessary for normal development. However, the increased incidence of tumours in these mice implies that p53 has a critical role in suppressing tumour growth, even in short-lived animals. From the study of human tumours, a related and unambiguous fact emerges: cells with inactivated p53 genes enjoy a selective growth advantage over their neighbours. The neighbours include neoplastic cells containing one or two normal p53 alleles, and the p53 mutant cells selectively proliferate, eventually expanding to form the dominant clone in the tumour.

So how does inactivation of p53 lead to this clonal expansion? Progress in this area depends on finding answers to the following questions. First, what induces p53 expression? In most cells, p53 is expressed at low levels, probably too low to exert a suppressive effect. In emerging tumours, *something* induces p53 expression to levels sufficient for growth suppression, and then (and only then) can an inactivating mutation of p53 endow a cell with a selective advantage, relieving the growth inhibition. Although exposure to high levels of radiation or other DNA-damaging agents has been shown to induce p53 expression in experimental systems, such high exposures obviously do not occur naturally. Second, is the p53-mediated growth arrest transient or permanent? Two different models for such growth arrest have emerged. In one, p53 mediates a transient G1 block, reversible after the p53 inducer is removed^{7,8}. In the other, the p53 'arrest' is permanent, resulting in apoptotic death⁹. These two models have distinctly different implications.

Third, what is the mechanism underlying the growth arrest? Three models have been formulated. In one, p53 may bind to and stimulate the expression of genes that are directly responsible for growth suppression, and p53 would then function primarily as an activating transcription factor¹⁰. In the second, p53 may inhibit the expression of transcription of TATA-controlled genes in general¹¹,

Table 2 Reported functions of wild-type p53

Biochemical functions

Binds DNA in a sequence-specific manner
Activates transcription from promoters with p53 DNA-binding sites
Represses transcription from a variety of promoters without p53 DNA-binding sites
Stimulates annealing of single-stranded DNA
Inhibits helicase activity
Inhibits DNA replication

Biological functions

Induces G1 growth arrest
Induces apoptosis following DNA damage
Inhibits tumour cell growth
Preserves genetic stability

perhaps by binding to TBP or other transcription factors (see Table 1). Finally, p53 may bind to cellular origins of replication, interacting with key proteins involved in DNA synthesis (such as RPA) and thus inhibiting entry into S phase (or, perhaps, directing cells primed for S phase into apoptosis).

Getting definitive answers to these questions will be difficult, but one clue is provided by p53 mutants. The mutants found *in vivo* have been selected, in the most relevant environment, for loss of p53 function(s) required for tumour suppression. In this regard, it is notable that many of the p53-associated proteins still associate with at least some mutant p53 proteins, and the simplest interpretation is that such interactions do not mediate p53 tumour-suppressive effects. Guests at the p53 inn will continue to come and go; such guests must be scrutinized by rigorous biochemical and genetic criteria before they are allowed to become permanent residents rather than friendly visitors. □

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Bendless delight

WATER pressure increases rapidly with depth, as divers know to their cost. A diver in deep water rapidly becomes saturated with pressurized air. If he returns too quickly to the surface, this air froths out of his blood and tissue like champagne when the cork is released. This is the dreaded 'bends'. Seals and dolphins breathe air as we do, yet dive and ascend as rapidly as they wish. How do they avoid the bends?

Daedalus recalls that bubbles like crystals, form in a liquid only in the presence of microscopic initiating 'nuclei'. Some arctic fish survive below freezing point by means of a protein inhibitor that deactivates the nuclei needed to form ice-crystals. So, says Daedalus, seals and dolphins must have evolved an inhibitor against air bubbles. It may deactivate the nuclei themselves, as alkalis do to the nuclei of steam bubbles (which is why alkaline solutions boil so irregularly). Or it may be surface-active, covering the bubble surface with a gas-impermeable monolayer which stops it growing. DREADCO biochemists are now analysing the blood of seals and dolphins in search of this natural bubble inhibitor. It will probably be a simple protein.

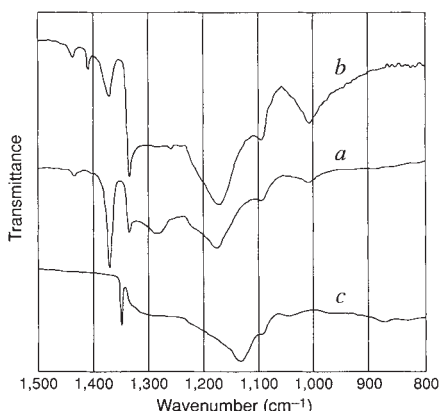
The diving fraternity will welcome the new product. One simple injection of DREADCO's Nobub[®], and a diver will be able to dive and ascend as freely as a seal or a dolphin. He will be able to shoot down to the depths, do his work, and shoot up again, without tedious delays for depressurization. But Daedalus is taking the idea further. Imagine, he says, a swimming bath whose water was itself loaded with Nobub. A compressor unit could saturate the water with tens of atmospheres of pure oxygen, and it would just stay in solution. It could be made available to swimmers by means of a mouthpiece or nasal spray, loaded with a Nobub-antagonist. Water entering the swimmer's nose or mouth would then fizz violently, disengaging enough oxygen for him to breathe. But Daedalus soon realized that the right amount of salt in the oxygen-loaded water would make it isotonic and fully compatible with human tissue, like medical saline. The swimmer could then simply breathe the water itself, as a fish does.

DREADCO's breathable Nobub bath will bring a wonderful new delight to its patrons. They will be able to float freely at any depth, buoyant and weightless, in warm water that they can breathe. Suspended so perfectly, they will enjoy the ultimate in total muscular relaxation. Even swimming lazily around will seem more like that euphoric, magical dream of flying.

David Jones

Errors in diamond synthesis

SIR — In 1955, some of us announced the first reproducible synthesis of diamond¹, details of which were subsequently published². These results marked the beginning of the present synthetic-diamond industry. But from the outset there were doubts in our team as to whether the first diamond grown by our technique (which we will call the run 151 diamond) was truly synthetic at all, or whether it was instead just a fragment of a natural diamond seed that got into the experiment inadvertently. We have now re-analysed the run 151 diamond using modern spectroscopic techniques and have found that it is indeed a small piece of a natural type Ia diamond.



Infrared absorption spectra of: a, run 151 diamond; b, a type Ia natural diamond with 'B'-form nitrogen; c, a typical synthesized diamond (type Ib).

In the early 1950s four of us (H. P. B., F. P. B., H. M. S. and R. H. W.), together with H. T. Hall, developed an approach to diamond synthesis at high pressures and temperatures. The pressure scale used in our experiments was Bridgman's 'resistance-jump' scale, which was suspected to be in error in absolute terms above 30 kbar or so. The proximity of our experimental conditions to the calculated graphite-diamond phase boundary was therefore uncertain.

The run 151 diamond appeared in an experiment using apparatus made of hard steel. According to the Bridgman 'resistance' scale, the pressure in this run was about 53 kbar, within the diamond stability field³. But later developments revealed that the true pressure could not have been much above 42 kbar, which is insufficient to stabilize diamond.

To investigate the true nature of the run 151 diamond, we recently removed it from the GE archives, cleaned it with acids and rinsed it with water. An infrared absorption spectrum was measured using an IR PLAN microscope attached to a Nicolet 740 FTIR spectrometer. The portion of

this spectrum shown in the figure resembles that of a natural nitrogen-containing type Ia diamond⁴. In particular, there are coincidences of the absorption bands at about 1,365 cm⁻¹ (related to nitrogen platelets), 1,330 cm⁻¹ (a Raman frequency, rendered infrared-active by defects and impurities), 1,280 cm⁻¹ (from nitrogen in the 'A' aggregate form) and 1,175 cm⁻¹ (from nitrogen in the 'B' aggregate form). We also show the spectrum of a typical nitrogen-containing synthetic type Ib diamond, which has characteristic bands at 1,130 and 1,343 cm⁻¹ (ref. 5); neither of these bands is seen in the run 151 diamond. We conclude that the run 151 diamond is a small piece of a natural type Ia diamond.

How the natural diamond got into the run 151 experiment is not clear, although it came to light only a week later, when the iron pellet from the run was being polished for metallographic examination. After we found this diamond and took it to be synthetic, Hall used a similar synthetic system of iron/iron sulphide/graphite in his 'belt' apparatus, which used a carbide piston and cylinder to achieve high

pressures⁶. This led to further successful runs and ultimately to the development of the process for synthesizing diamonds at high pressures and temperatures from graphite reacted with molten group VIII metals and alloys, which we described fully in 1959² (after a US Department of Defense secrecy order had been lifted). Our mistake was therefore clearly a most serendipitous one, as it provided the impetus to experiment with that system at higher pressures, leading quickly to the "right" and "reproducible" results.

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Galaxies and magnetic fields

SIR — The observed flat rotation curves of spiral galaxies constitute one of the most important facts suggesting the existence of large amounts of so-called dark matter in the Universe. However, if the galactic gas could be held in equilibrium by forces other than gravity alone, then an important argument for excess galactic mass would be weakened. Battaner *et al.*¹ suggest that stresses from an azimuthal magnetic field at the peripheries of a galactic disk can provide the confining stress needed. Here we reconsider the question of magnetic-field and gas equilibrium, and point out that the simple success of the one-dimensional Battaner *et al.* model depends on their incomplete treatment of the three-dimensional equilibrium. Application of the well-known virial theorem shows that the addition of magnetic field to their system would require more dark matter to maintain equilibrium, not less.

The pertinent steady-state virial theorem is^{2,3}

$$2(T + T_t) + M + \int_V d^3r x_i F_i = S \quad (1)$$

where $T = \int_V d^3r \rho v^2/2$ is the sum of directed kinetic energies; $T_t = \int_V d^3r \rho \langle u^2 \rangle/2$ is the sum of thermal or random kinetic energies; $M = \int_V d^3r B^2/8\pi$ is the total magnetic field energy, and F_i is the force of gravity. $S = \int_V d^3r x_i \{-p\delta_{ik} + M_{ik}\}$ is the surface stress integral, with p the particle

pressure and M_{ik} the Maxwell stress tensor.

Following Battaner *et al.*, consider an isolated system with $S = 0$. The terms T , T_t and M in equation (1) are positive definite; only the last term on the left of the equation, the gravity integral, is negative. Regardless of the configuration or geometry, in the overall dynamical balance of a physical system, magnetic field is an expansive stress. Equation (1) shows explicitly that the only confining stress for large, isolated, astrophysical systems is gravity.

Applying equation (1) to the specific situation addressed by Battaner *et al.*, we first consider the case in which magnetic field stresses can be neglected ($M \approx 0$). Focusing only on the super-keplerian gas — treating it as an isolated system immersed in external gravity — it is clear that virial equilibrium requires a dynamical balance between the expansive tendency of the gas motions and the confining influence of gravity acting on that gas. The need for dark matter is already encapsulated in this analysis.

Now, with everything else unchanged, assume that the gas is magnetized. According to Battaner *et al.*, assuming a special field configuration, the magnetic stresses should take up some of the expansive tendency of the gas motion, so that the strength of gravity needed diminishes. But, from the more general virial rela-

tionship described by equation (1), because M is always positive, it is immediately clear that, rather than resulting in a smaller F_i , the addition of magnetic field increases the needed magnitude of F_i . This implies the presence of even more unseen mass than in the unmagnetized case. Thus the addition of a magnetic field does not alleviate the dark matter problem; in this simplest case it exacerbates the problem.

The preceding analysis, couched in terms of an isolated (except for gravity) system, with $S = 0$, is that considered by Battaner *et al.* Now we turn briefly to the full virial equation, including the surface stresses contained in S of equation (1). The simple consequences, derived in the previous paragraph, of the virial relationship applied to the peripheral gas could be evaded by magnetic or pressure stresses, S , transmitted across the bounding surface.

This could be accomplished by a magnetic field anchored to a massive central object, but this may be shown to lead to a very large required field, contrary to observations (J. R. J. and E. H. L., manuscript in preparation). Alternatively, the expansive magnetic stress may be confined by the pressure of an external medium. A confining magnetic field would have to be a cosmological entity as strong as the confined magnetic field itself, filling the entire Universe, otherwise dynamical problems of confinement, similar to those described above, would simply arise on a larger scale. Or one could imagine high-pressure intergalactic gas (or cosmic rays) providing the pressure to confine the magnetic field. In either case the external confining pressure would have to be as high as the confined pressure to provide the needed virial equilibrium.

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SIR — Battaner *et al.*¹ argue that the flat rotation curves of galaxies such as M31 may be caused not by dark matter, but by magnetic tension. In essence they suggest that the centripetal force required to hold together a rapidly spinning disk of gas is provided by the tension of an azimuthal magnetic field B_ϕ rather than by the inward gravitational attraction of dark matter. Here we show that a field strong enough to alter significantly a galaxy's rotation curve will cause the gaseous disk

to flare unacceptably.

Because gas pressure makes a negligible contribution to the equation of horizontal equilibrium, the circular speed v_c , the streaming velocity of gas v_ϕ and the magnetic field B_ϕ are coupled by

$$v_\phi^2 - v_c^2 = \frac{1}{2\mu_0 R^2 \rho} \frac{\partial}{\partial R} (R^2 B_\phi^2) \quad (1)$$

where R is the galactocentric radius and ρ is the gas density. The magnetic field imposes two sorts of forces: azimuthal tension and pressure in both the radial and the vertical directions. The tension always imposes a net inward force, and thus tends to increase v_ϕ . The radial pressure force $\propto \partial B_\phi^2 / \partial R$ acts inwards or outwards depending on whether B_ϕ increases outwards or inwards. When $B_\phi \propto 1/R$, the radial forces arising from tension and pressure balance exactly and $v_\phi = v_c$.

As Battaner *et al.* remark, the field strength plotted in their Fig. 1 asymptotes to this form at large R . Consequently, this field makes a negligible contribution to v_ϕ , contrary to the claims of Battaner *et al.* (see our Fig. 1).

We find that to make a field of the order

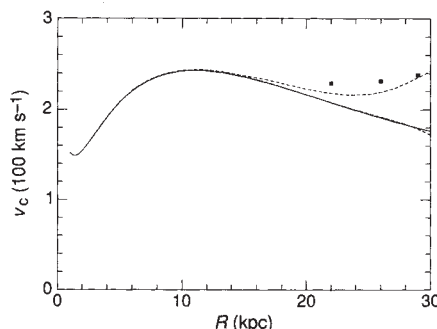


FIG. 1 Circular speed of Kent's maximum disk model² of M31 (full curve) and v_ϕ calculated from $B_\phi(R)$ as given in Fig. 1 of ref. 1 (lower dashed curve). The upper dashed curve shows $v_\phi(R)$ for a model in which the B_ϕ is twice as large as in ref. 1 for $R < 15$ kpc, and constant for $R > 15$ kpc. Data points from ref. 3.

of $B_\phi \approx 10 \mu\text{G}$ advocated by Battaner *et al.* dynamically significant, the asymptotic magnetic pressure must be directed outwards with the result that both it and the tensile force tend to increase v_ϕ (Fig. 1).

Magnetic pressure thickens the disk vertically by pushing gas upwards with a force $\propto \partial B_\phi^2 / \partial z$, which vanishes only in the totally implausible case in which B_ϕ^2 does not fall off with increasing z . In reality one expects B_ϕ^2 to fall off roughly with the gas density ρ . In this case the vertical pressure

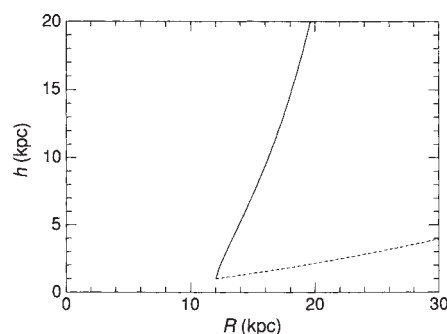


FIG. 2 Scale height $h(R)$ in the M31 model of ref. 1 if the rotation curve is held constant in the keplerian regime by a magnetic field (full curve) or is allowed to drop as it would in the absence of a magnetic field (dashed curve). The mass $M = 10^{11} M_\odot$ has been inferred from the measured luminosity $L_B = 2.5 \times 10^{10} L_\odot$ (ref. 2) and mass-to-light ratio $\gamma_B = 4$ (refs 4, 5). Disk scale length is $R_d = 4.3$ kpc as in ref. 1.

force is at least an order of magnitude greater than the radial tensile force $\sim B_\phi^2/R$, as $R \gg h$, the disk scale-height.

Figure 2 confirms this qualitative analysis by plotting $h(R)$ for $R \geq 12$ kpc as derived for M31 from the field strength shown in Fig. 1 of Battaner *et al.* from equation (1) above. Specifically, we have taken the gas to be isothermal with $B_\phi^2 \propto \rho$, and solved equation (1) for $B_\phi^2(R, 0)$ under the Battaner *et al.* assumptions of a constant circular speed $v_c = 240 \text{ km s}^{-1}$ and a keplerian mass distribution. The gas temperature is implicitly determined by requiring $h(12 \text{ kpc}) = 1 \text{ kpc}$. Once $B_\phi^2(R, 0)$ is known, $h(R)$ follows immediately. For comparison, the dotted curve shows the variation of the scale height expected in the absence of a magnetic field for one choice of $h(12 \text{ kpc})$.

The upward surge of the full curve in Fig. 2 demonstrates that a magnetic field large enough to change a keplerian rotation curve into a flat one will cause the gas disk to flare unacceptably. The origin of this thickening is simply that a magnetic field provides pressure in two directions and tension in only one. If the field's azimuthal tension is dynamically important, its pressure will blow the disk apart vertically. We have demonstrated the violence of this effect in only one illustrative case, but it is intuitively clear that the effect is a perfectly general one.

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of fewer than 500 words and five references.

Shared structural motif in proteins

SIR — Swindells *et al.* in Scientific Correspondence¹ observe that the phosphocarrier protein HPr shares with acylphosphatase (APt) a structural motif made of three antiparallel β -strands and two connecting α -helices. They ask whether this is a recurrent phosphate-binding motif. The answer is no.

Alpha/beta motifs are usually built around a parallel β -sheet, but antiparallel β -sheets are not uncommon. Ferredoxin² is an early example of the antiparallel α/β fold. Its four-stranded β -sheet has the same topology as in APt³, the activation domain of procarboxypeptidase⁴ and the RNA-binding domain of the U1 ribonucleoprotein⁵, but HPr (ref. 6) is different. When we determined the X-ray

there are twelve topologies; eight can accommodate helix connections. Second, our comparison is based on known positions of the binding sites, whereas that of APt is unknown. The NDPK active site is a histidine which becomes phosphorylated as part of the catalytic cycle. It is on the face of the β -sheet that is not covered with α -helices. So are the nucleotide-binding site of the allosteric domain⁸ and the RNA-binding site of the U1 domain as defined by mutation studies⁵.

I predict that APt will have its active site on this face of the β -sheet rather than near helix $\alpha 1$ as in HPr. Part of the HPr β -sheet can be superimposed on NDPK just as for APt (see figure). The fit is excellent for the three common β -strands, but very poor for α -helices, and the remainder cannot be superimposed at all. Both NDPK and HPr carry phosphorylated histidines. They are 28 Å apart, about as far as possible in proteins of this size. In my view, this precludes the common three-stranded structural motif from having a functional significance.

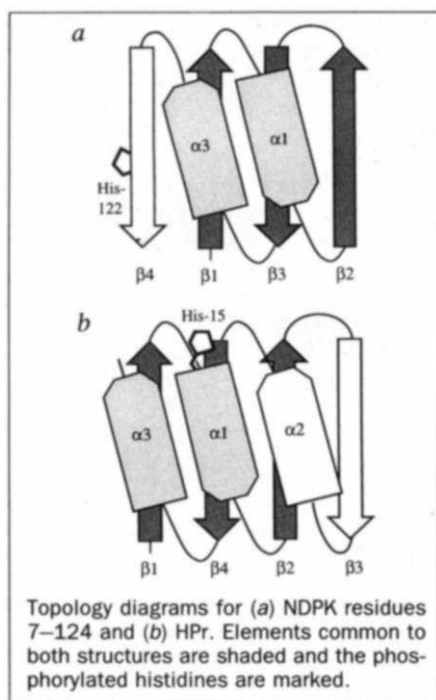
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structure of nucleoside diphosphate kinase (NDPK)⁷, we found that the NDPK subunit is like all these proteins, and also like the allosteric domain of *Escherichia coli* aspartate transcarbamylase⁸, which binds nucleotides.

We suggested⁷ that the four-stranded antiparallel α/β structure of NDPK is a nucleotide-binding motif, different from the one based on a parallel β -sheet found in most kinases⁹. I think that this proposal has a stronger basis than that of Swindells *et al.*¹. First, the fourth strand makes the motif less likely to occur by chance. With three strands, the β -sheet can have only three different topologies, two of which accept α -helix connections. All antiparallel α/β structures contain one of these two topologies. With four β -strands,

figure). This is important because in NDPK these helices form a cleft on one side of the sheet into which the nucleotide binds. Unfortunately, current structure and sequence data are inconclusive and only experimental work will accurately pinpoint the active site of APt.

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Bird or dinosaur?

SIR — Perle *et al.*¹, in reporting their find of the fossil *Mononychus*, call it a 'bird' in the title of their paper and a 'dinosaur' in its third and fourth lines. They endorse the view² that *Archaeopteryx*, although a bird, is not a member of Aves. Instead, *Archaeopteryx* is a member of Aviales^{1,2}, a more inclusive taxon than Aves that now also includes the taxa *Ornithurae*³, *Metornithes*¹ and *Ornithothoraces*³. Each of these new taxa differs from Aves only by including one or more fossil species; when new fossils are discovered or the relationships of known fossils are analysed with greater care, many more such taxa will surely be necessary. Hennig^{4,5} foresaw the problem: "This kind of procedure would lead to unimaginable nomenclatorial complications — and in some cases has already done so"⁵.

Hennig^{4,5} showed that there are three ways of circumscribing a monophyletic group with living and fossil members. The first (method 1) is to limit it to the species that "appear to have descended from the latest common stem-species"⁵; this, the 'crown-group'⁶ concept, is adopted by Perle *et al.*¹ in circumscribing Aves. The second (method 2), the 'total-group'⁶ concept, is to include all fossil species that are more closely related to the crown-group than to its extant sister-group. The third (method 3), which Hennig called "the method usually followed in palaeontology", is to select one or more of the characters of a group as "essential", and to include in the group only those fossils possessing the key character(s); this method is used by Perle *et al.* in circumscribing 'birds', with feathers as the key character. Hennig settled on method 2 as "the most suitable one for phylogenetic research". Perle *et al.* have chosen a combination of methods 1 (in circumscribing Aves) and 3 (in circumscribing 'birds') which, apart from the nomenclatorial complications that method 1 demands,

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causes confusion among those trying to interpret their results. Is *Mononychus* a bird, a dinosaur, a bird-like dinosaur or a dinosaur-like bird? For Perle *et al.*, judging by the title and first few lines of their paper¹, it is a member both of birds (a monophyletic group) and dinosaurs (a nonmonophyletic group). For journalists, 'dinosaur' is more interesting than 'bird', and so we get 'bird-like dinosaur', just as the extinct stem-group mammals were long known as 'mammal-like reptiles'. Both usages are falsely inverted, giving the strength of the noun to the non-group (dinosaur, reptile) and the weakness of the adjective to the natural group (bird, mammal); 'dinosaur-like bird' and 'reptile-like mammal' correctly indicate inferred relationships.

In mammals, the proliferation of names for stem-group taxa that Hennig anticipated is now manifest^{7,8} because, as with birds, restriction of the name Mammalia to the crown group is being advocated^{9,10}. Asked when mammals first appeared, a supporter of this procedure might respond with an age ranging over about 160 million years¹¹, from early Cretaceous, the inferred age of the earliest monotremes and therians (method 1), through a range of intermediate dates (method 3)¹² to the late Carboniferous (method 2), when the mammals are inferred to have separated from their Recent sister-group. For birds, a similar, though less extensive, range of dates might be offered. Advocates of methods 1 or 3 may thus provide misleading responses. The molecular biologist, for example, does not want to know when monotremes or ratites first appeared

(method 1), or when ear ossicles or feathers first appeared (method 3). Instead, he or she wants to know when mammals (or birds) separated from their Recent sister-group (method 2) as the basis for estimates of rates of molecular evolution or for calibration of molecular clocks. Hennig favoured method 2 for theoretical and practical reasons; within that method, there are strategies that avoid proliferation of names for stem-group taxa^{13,14}. Surely it is time we palaeontologists agreed to avoid further needless proliferation of names and misleading inferences, and settled, with Hennig, on his method 2.

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does not show a correlation with the G1 checkpoint⁹.

AT cells are similar to caffeine-treated cells in that they are highly sensitive to ionizing radiation and show reduced cell cycle delays in G1, S and G2 phase¹⁰. As with caffeine-treated cells, the absence of a radiation-induced G1 delay in AT cells is associated with a failure to induce p53 protein². Although abnormal cell-cycle regulation was originally proposed as the mechanism of radiosensitivity in AT (ref. 11), several lines of evidence suggest that this is not the case. (1) Delaying cell-cycle progression in AT cells does not enhance survival after exposure to ionizing radiation¹²; (2) AT cells show increased chromosome damage immediately after ionizing radiation exposure without progression through the cell cycle¹³; and (3) the radiation hypersensitivity and abnormal cell-cycle delay in AT are separable phenotypes^{14–16}.

Studies with transformed cell lines also indicate a minor role for p53 and the G1 checkpoint in radiation sensitivity. SV40-transformed cells, in which T antigen inactivates p53 (ref. 17), do not show increased cell killing after ionizing radiation¹⁸. Further, the variability in cell survival in human tumour cells in response to ionizing radiation^{19,20} does not correlate with the presence of p53 mutations²⁰. Sensitivity to radiation-induced chromosome aberrations after exposure of tumour cells in G1 phase is also apparently independent of p53 (ref. 19).

In view of these studies, it seems highly unlikely that the genomic instability associated with p53 mutations is due to the cell's failure to repair DNA damage before attempting DNA replication. Genomic instability must therefore result from other complications of defective cell-cycle regulation or one of the many other cellular pathways affected by p53 mutations.

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Cell checkpoint and radiosensitivity

SIR — Recent studies highlighting the role of p53 protein in cell-cycle regulation have heightened interest in the importance of cell-cycle checkpoints in mammalian cell responses to DNA damage. Cells with altered or absent p53 protein show increased genomic instability¹ and, after exposure to ionizing radiation, a much reduced delay in the G1 phase of the cell cycle^{2,3}. It has been suggested that the increased genomic instability results from the reduced G1 delay and the cell's subsequent failure to repair DNA damage before it attempts DNA replication in S phase⁴. This sensitivity to DNA damage should result in greater frequencies of radiation-induced cell killing and chromosome damage. However, evidence from studies of cells treated with caffeine, cells derived from patients with ataxia-telangiectasia (AT), transformed cell lines and tumour cells strongly suggests that the G1 checkpoint plays little or no role in determining the sensitivity of mammalian cells to ionizing radiation.

Caffeine treatment reduces ionizing

radiation-induced cell-cycle delay, affecting cells in the G1, S and G2 phases of the cell cycle^{2,5}. Caffeine seems to stabilize factors required for progression through the cell cycle⁶, and inhibits induction of p53 after radiation exposure². Caffeine treatment can enhance radiotoxicity; however, this occurs primarily during G2 phase⁷ and does not show a correlation with reduced cell-cycle delay⁸. In addition, the timing of the caffeine-induced increase in chromosome aberrations in normal human lymphocytes

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Solid beginnings

David Shoenberg

Out of the Crystal Maze: Chapters from the History of Solid-State Physics. By L. Hoddeson, E. Braun, J. Teichmann and S. Weart. *Oxford University Press*: 1992. Pp. 697. \$75, £50.

IN the presentation of a completed piece of original research, the author usually aims to be as logical and concise as possible and so omits any hint of the sometimes complicated history of how his or her results were really achieved. If the work proves later to be important enough to merit mention in a textbook or a course of lectures, this omission may be unfortunate because it is just such omitted anecdotes and historical sidelights that can illuminate the story and make it memorable for the student. And, of course, the inner story of how the author really worked things out may provide useful raw material for the historian.

During the 1970s, an international group of historians of science, led by Lillian Hoddeson and Ernest Braun, felt the time was ripe to embark on a history of solid-state physics, which over the previous 50 years had grown from small beginnings to a fairly well defined and widespread discipline of its own. To avoid losing raw material that might otherwise disappear with the passage of time, they have tape-recorded interviews with as many as possible of the scientists who made significant contributions up to about 1960, the end of the period they intended to cover. As the editors say in their preface: "there is nothing like the oral history for giving a dead body of facts something akin to a soul". To produce a coherent account of what really happened, they have woven this oral material into the considerable archival and biographical material left by those no longer alive, while also drawing, of course, on the original scientific papers.

The title *Out of the Crystal Maze* is aptly chosen, for the history of science is indeed a maze and solids are mostly crystalline. The maze is an intricate one with a complicated mix of false trails, serendipity, interplay between science and technology, interplay between theory and experiment and sociological influences. Extracts from the recorded recollections of those personally involved provide lively and illuminating clues to finding a way through the maze. But here a warning is relevant (as the editors of the book are aware). The recollections are mostly of events that happened long ago and memory is not always reliable—it is sometimes slanted, quite innocently perhaps, to enhance the importance of a scientist's own contribution. A difficulty in presenting the vast amount of material that has gone

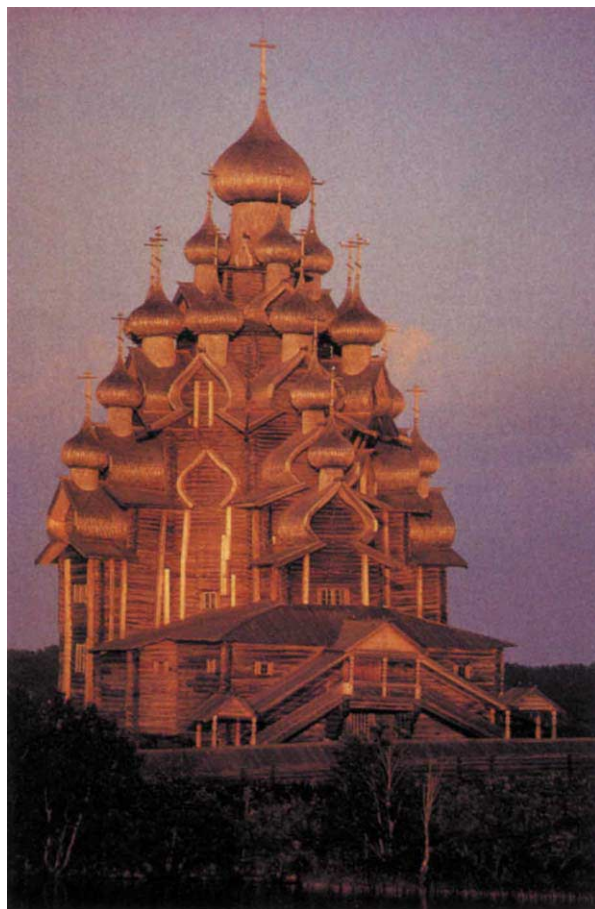
to build up a coherent picture of any subdivision of the subject is to strike the right balance between explaining the physics of each particular development and merely giving headings. On the whole, the authors have steered reasonably successfully between the Scylla of going too far into the physics and the Charybdis of producing a mere catalogue of topics and names of people. If anything they have steered too close to Charybdis—at least, I often felt I needed more than just a heading to appreciate the relevance of some of the contributions mentioned, especially in areas with which I was less familiar.

An early chapter on the application of quantum mechanics to the interpretation of the properties of solids is particularly interesting in showing how an almost explosive growth was nurtured by the close interaction of a relatively small group in Germany. These were students

of Sommerfeld, Born, Heisenberg and Pauli (who later settled in Switzerland) and they also drew inspiration from Bohr in Copenhagen. There are many amusing anecdotes. I particularly liked Pauli's characteristic comment: "I don't like this solid state physics . . . though I initiated it".

This golden era lasted for less than 10 years and ended in 1933 when Hitler came to power and many of the active participants, being Jewish, had to emigrate. This emigration proved eventually to be much to the benefit of the West, for it led to a great flourishing of theoretical physics under men such as Bethe, Peierls, Bloch and Weisskopf. The chapter on band theory continues the same quantum theme as applied to the behaviour of electrons in metals, but the complicated story of the many stages in the improvement of the theory to its present high level is not easy to follow. And although there are entertaining recollections about the experiments that provided checks of the theory, the relation between theory and experiment is not clearly brought out.

Other chapters, on defects, mechanical properties, semiconductors, magnetism and collective phenomena, cover particular aspects in depth but contain significant gaps. For instance there is a detailed history of the early development of



WORSHIP in wood — the Church of the Transfiguration of the Saviour on Khizi Island, built in 1714 in honour of Peter the Great's victories over the Swedes, is the supreme example of Russian wooden architecture. Construction was done without clay caulking and nails, relying instead on the tight fit of one log above another, with moss and hemp used for insulation. The elaborate superstructure provided an efficient system of ventilation to prevent the logs from rotting. This picture is one of hundreds from *A History of Russian Architecture* by William Craft Brumfield, an immense survey ranging from the masonry churches of tenth-century Kievan Rus to the prefabricated buildings of the present. CUP, £75, \$95.

magnetism in France, under the leadership of Curie, Langevin, Weiss and Néel, with a fascinating account of the rise and fall of the "Weiss magneton", but the later developments are only rather summarily dealt with.

A final chapter on the solid-state physics community attempts to monitor, by using statistical analyses, the growth of solid-state physics as a distinct discipline. This seems to me rather a sledgehammer approach. What is perhaps underemphasized is that the community is a loose one, with subcommunities such as those studying low-temperature physics, superconductivity, magnetism, semiconductors and crystallography. These subcommunities hold their own conferences and publish their own journals and in practice have little overlap with each other.

As the subtitle "Chapters from the History of Solid-State Physics" implies, the coverage is only partial and many topics remain to be dealt with. But it is regrettable that there is so little about the important school of solid-state physics in the former Soviet Union, particularly as the advent of *glasnost* in the 1980s should have made it possible to include more. I shall mention only one example. Although Douglas Hartree's contribution to the Hartree-Fock theory is extensively discussed, that of Vladimir Fock, one of the greatest Russian theoreticians, is relegated to a footnote. Other shortcomings indicate inadequate editing and proof-reading. The policy of using first names of people when they are first mentioned is not carried through consistently. It grates a little to read "Bertram Brockhouse and A. T. Stewart" as if Alec Stewart was not important enough to deserve a first name. But worse is that some first names are simply wrong. To call Mike Garfunkel Michael (it should be Myron) is perhaps excusable, but Douglas instead of Dermot Roaf and Israel instead of Evgenii Lifshitz is a bit too much. The photographs of leading participants help to provide intimacy between the reader and the scientists, but their value would be enhanced by better captions with more details of time and place. Here too there are mistakes: for instance the captions for Bardeen and Seitz are, I think, interchanged and the picture of Pippard and Kapitza "in Cambridge, early 1960's" was in fact near Moscow in 1957.

This is nevertheless a valuable start to an ambitious project and the book is likely to become an authoritative secondary source for anyone trying to follow up some aspect of the history in depth, so it is to be hoped that there will be a new and enlarged edition in which the shortcomings I have mentioned will be avoided. □

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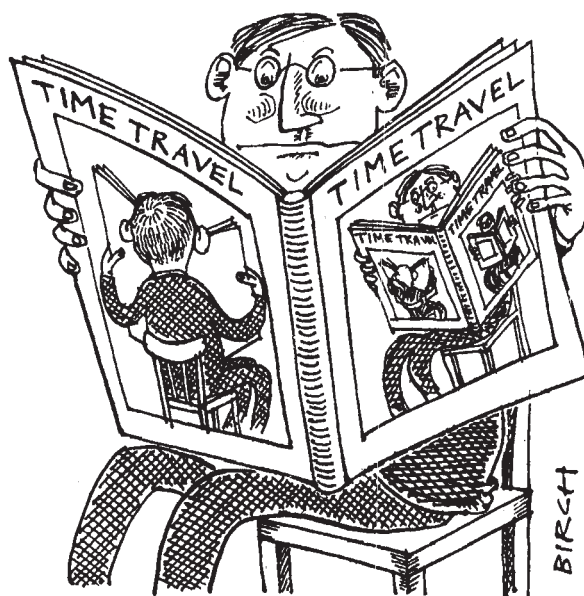
Against the clock

Ian Moss

Time Machines: Time Travel in Physics, Metaphysics, and Science Fiction.

By Paul J. Nahin. AIP: 1993. Pp. 408. \$45, £40. (Distributed outside North America by OUP.)

H. G. WELLS has a marvellous description of time as the "fourth dimension" in his novel *The Time Machine*; it is all the more impressive for being written as long ago as 1895. He had probably been reading *Nature*, for in a July issue of that year there sits an account of an address on the fourth dimension by Simon Newcome, and Wells



mentions the address in his story. This kind of detail fills every paragraph of *Time Machines*.

But what about time travel as more than just a plot device? To retain some respectability we need to talk about 'chronology violation' rather than time travel. In the theory of general relativity, chronology simply means the time ordering of events along lines in spacetime. It is as much a physical concept as force or velocity are physical concepts in newtonian mechanics. (General relativity is Einstein's theory of gravity that seems to fit all of the available data best.)

Work on chronology violation has by necessity taken the form of thought experiments. If one tries to answer the question "what happens if?" by using the theories available at present, then one starts to find inconsistencies that suggest how those theories might have to be modified. If all goes well, this enables one to plan real experiments that test the new theories.

Kurt Gödel, the outstanding mathematician and philosopher, gave the first example of a model universe that was

perfectly consistent with general relativity but violated chronology. In Gödel's universe, a spaceman could fly off in a rocketship and eventually rejoin her own past. (Ordinary English grammar assumes a chronology that is not adequate in this context.) Such a journey through spacetime describes a 'closed timelike loop'.

With the introduction of black holes it becomes possible to find many more examples of closed timelike loops. Instead of being laid down like a sheet, spacetime can have a complicated structure with tubes and folds connecting distant times and places. These often involve new types of matter called 'exotic', which may come in the form of particles that travel faster than light, fields with negative energy or infinitely long strings.

Some issues of *Physical Review* are full of exotic matter, but it is still an open question whether exotic matter is necessary.

In many cases, the quantum mechanical energy of the vacuum seems to prevent causality violation, leading Stephen Hawking to propose a 'chronology protection conjecture'. If this conjecture is false then there would be causality violation paradoxes. An example is the grandfather paradox: what would happen if a time-traveller went back in time and tried to kill his own grandfather? There is also the causal loop paradox: a time-traveller goes back in time

to tell his younger self how to build a time machine. Where does the information about how to build a time machine come from? These paradoxes are now studied in mathematical physics and no longer seem to be the obstacles that they were once thought to be.

The research that has gone into this book is impressive. The author has made a good selection of ideas from the scientific literature on spacetime, causality violation and time-travel paradoxes, and they are presented at a popular level, with science-fiction plots running in parallel. Often the fictional ideas came first. Bob Olsen's doggerel, for example, which appeared in the July 1934 issue of *Amazing Stories*, is as pertinent today as it was then:

Let's hope, in planning new inventions,
They'll give us cars with four dimensions.
When searching for a parking place
We sure could use some hyperspace! □

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Past masters

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Getting Here: The Story of Human Evolution. By William Howells. *Compass*: 1993. Pp. 261. £26 (hbk); £14.50 (pbk). (UK distribution, Bailey.)

IN the library of my old medical school, books on evolution were next to the anatomy section. When I grew weary of the brachial plexus or failed once again to appreciate the nuances of cardiac development, I would often browse through them. Few new volumes were ever added, but one that did appear was *Mankind in the Making* by William Howells. I read it from cover to cover and it was probably one of the reasons I strayed from the path of medical righteousness and became a student and then teacher and researcher of human evolution.

Medical students are not renowned for the depth of their literary appreciation, and I was no exception. But even I could recognize a writing style that suggested that rare combination of a learned man who wore his learning lightly. The style was direct, authoritative and engaging, the antithesis of patronizing. It was not until much later that I realized that Howells's literary style was probably the product of both nature and nurture. Harvard contributed the nurture and a grandfather, a writer by the name of William Dean Howells, undoubtedly supplied the nature.

The human evolutionary history that Howells so infectiously recounted back in the early 1960s was a relatively simple story based exclusively on fossil evidence. Nearly all forms of extinct hominids were located, at one place or another, in a sequence that led to modern humans. Molecular anthropology had yet to make an impact on the study of human evolution, and morphometry was only just being exploited as a tool to measure the similarity in form between fossils. In the event, no-one has subsequently used multivariate morphometrics to better effect than Howells. Over many years he has accumulated a unique dataset of measurements of modern human crania. These data include all the principal geographical variants of *Homo sapiens* and their interpretation has been the focus of Howells's research for the past two decades.

It is a reflection of the quality of this research that such a potentially contentious endeavour has been undertaken without controversy. In his most recent monograph, "Skull Shapes and the Map" (Papers of the Peabody Museum, No. 79, Harvard University, 1989), Howells added 10 cranial series to his original 18 and considered patterns of variation with-

in and between the 28 samples. The research was designed to probe the link between genes and phenotype. To what extent are variations in modern human cranial form determined by 'deep' evolutionary history as opposed to relatively recent environmental selection pressures? Evolutionary trees, or dendrograms, based on cranial forms are then compared with those generated using the observed distribution of genetic markers, including mitochondrial DNA.

In studying the origin of modern humans, any attempt to analyse and interpret the fossil evidence should try to correct for the effects of differences in temperature and humidity on the phenotype of the modern human cranium. But few workers make this effort. It is no coincidence that nearly all those now assessing the evolutionary implications of modern human cranial variation do so using Howells's database, which is freely and generously made available to those who request it. Howells's recent study provided no support for the regional continuity hypothesis, which states that, post-*Homo erectus*, 'vertical' local continuity was a much stronger influence than 'horizontal' gene flow between contemporaneous populations. But nor did it provide compelling evidence to support a sub-Saharan origin for anatomically modern humans.

Now well into his 80s, Howells has once again written a survey of human evolution. The title *Getting Here* and the use of the word 'story' in the subtitle are, I suspect, a gentle dig at those who regard all attempts at presenting human prehistory as variations on a folklore narrative, in which the hero triumphs over adversity. The book is a narrative account of human evolution, but Howells makes it abundantly clear that human evolution was neither preordained nor inevitable. He uses all the benefits of a narrative to carry the reader through the confusing maze of fossil evidence. Howells has been successful in his attempt to explain to the general reader how palaeoanthropologists go about the task of reconstructing human evolution. The book concentrates on morphological evidence, and although it sets some of that evidence in its environmental context, neither ecology nor palaeoecology appears in the index.

Earlier accounts of human evolution, including those of Howells, stress the linearity of the human family tree; the tree appears more like a telegraph pole than a bush. In his latest account, Howells suggests that hominid evolution is best seen as series of 'bush-like' adaptive radiations. Within each radiation there is a potentially confusing mosaic of dietary, locomotor and other behavioural adaptations and abilities. Thus, for example, bipedalism and encephalization — the acquisition of a relatively large brain — may have

evolved more than once. Howells's comprehensive treatment of the origin of anatomically modern humans is particularly good. It eschews the more confrontational style of much of the 'regional continuity' versus 'out of Africa' debate and, instead, takes the trouble to tell the reader how he or she might go about assessing the evidence.

If the potential for wealth creation is to become an all-powerful influence on science policy, then much of palaeontological science is doomed. But if public interest is to be the criterion, we are a long way from slaking the public's thirst for knowledge about our past. Howells's new book is a balanced and intellectually rigorous account of human evolution that is mercifully free from partisan adherence to a particular evolutionary picture. To an author well into his eighty-fifth year, the title has a special significance. We must be grateful that Howells's deep appreciation of human evolution has been updated and made available to a general audience. He tells us that this really is his final performance: if so, we shall miss the clarity of his analysis and the elegance of his writing. □

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Baby talk

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The Child's Path to Spoken Language. By John L. Locke. *Harvard University Press*: 1993. Pp. 518. \$39.95, £27.95.

JOHN Locke, the philosopher of mind, might have been surprised at the pre-linguistic abilities of the young infant that are enumerated here by his twentieth-century namesake. Neonates appear to be able to mimic an adult's facial gestures soon after birth — even before they have seen their own face. And two-month-old babies are sensitive to the difference between sounds such as *b* and *g*, even though this contrast will not be used linguistically until at least a year later. The original Locke would also have been impressed by the detailed and careful sifting of empirical evidence in this account of the infant's first year or so of linguistic life.

Current accounts of the development of language, whether they are concerned with normal or abnormal development, tend to concentrate on the period after the first recognizable use of words: that is, on the child's transition from words to grammar and the subsequent development of grammatical capacity. The dominant view has been that language is a uniquely

human achievement, and that what distinguishes language from the communication systems of our primate cousins is grammar. Grammars of natural languages signal grammatical relations, using word order or cases. They have tense endings, agreement markers, gender, distinctions between singular and plural, personal pronoun systems and so on. Most human infants learn these complex systems perfectly, without apparent effort. Research has concentrated more on the form of the infant's acquisition of the abstract linguistic code than on comparisons with non-human primates, precursors to language in the child's very early development, or the biological or social context of language development.

Locke's aim is to redress this balance by close scrutiny of the first year of life. In a fascinating, scholarly and clearly written account, he takes us to the brink of language proper by examining its perceptual, social, neural and cognitive precursors from before birth to the appearance of the first recognizable words. What is it about the human infant that makes it different from the young nonhuman primate? And what is it about the strands of early development, and the social context in which they are embedded, that guarantees that all but a small percentage of human infants are eventually able to speak the language of their environment?

Much of the material brought together in the book — the development of the child's early ability to recognize faces and voices, the development of perceptual capabilities, vocal behaviour and neural capacity — is not new. What is original is the unifying perspective from which Locke synthesizes a mass of information from his own and others' previous research. The question he wishes to address is how a child comes to speak. This ontogenetic equivalent of a phylogenetic question that we can never answer demands a systematic review of the development in the child's first year of a "variety of language-related mechanisms [which] will inch their way toward efficient action". As a source of information on these mechanisms, this book would be difficult to better. But a detailed description of the increasing complexity of the child's vocal behaviour, its developing perceptual abilities and capacity for reference does not fully account for the fact that children learning any language begin to use words from their parents' lexicons at or about the time of their first birthdays.

The author's explanation for the infant's motivation to speak fully acknowledges the role of language as a medium for social and emotional interaction. Language expresses affect (emotion associated with an idea) through prosody (patterns of stress and intonation), while also expressing propositions through

words and their grammatical organization. Locke believes that these dimensions of language, the "warmly emotional" and the "coldly analytical", are equally important. Indeed, for the infant, the affective dimension may well be initially more important. Locke argues that it is the infant's social cognition, in relation to what is said to him or her, that plays a "precursive and enabling role" in the development of spoken language. This (possibly specialized) capability is seen, for example, in the neonate's orientation to face-like stimuli a few minutes after birth, the gazing of infant and mother at one another, and early infant preferences for the mother's voice and prosody. The infant therefore does not 'acquire' language but is drawn towards it by his or her active desire to communicate with caretakers, and by their reciprocal role in socialization. The specialization in social cognition, some

aspects of which are shared with nonhuman primates, delivers the infant to the point at which the other aspect of our species' dual specialization for language, the grammatical analysis module, can contribute.

While impressively developing his thesis, Locke is careful to bring the child only as far as the 'doorstep' of theories about the acquisition of the abstract linguistic code. There is little doubt that his bio-linguistic perspective on the precursors of linguistic development will influence researchers working on that subject. I would also hope that his work will encourage researchers examining early grammatical and phonological development to consider seriously the biological basis of these aspects too. □

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Warm or cold environments?

Eric J. Barron

Climate Modes of the Phanerozoic. By L. A. Frakes, J. E. Francis and J. I. Syktus. Cambridge University Press: 1992. Pp. 274. £40, \$69.95.

CLIMATE Modes of the Phanerozoic is a review of the Earth's climate over the past 600 million years. This text is an update of an earlier work by L. A. Frakes, *Climates Throughout Geologic Time* (Elsevier, 1979). There are some important differences. Whereas the earlier text examined climate as a function of stratigraphic subdivisions of time, the organizing basis of the new text is the warm and cold climate 'modes' of the Phanerozoic. The authors emphasize the climatic extremes recorded in rocks and sediments and describe the entire record as sets of either cold or warm climates. The new volume is also strengthened by the addition of material on the terrestrial climate record and a greater emphasis on Quaternary climates.

There is little discussion about the continuing controversy over climate forcing factors, modes or the characteristics of past climates. The book is primarily an interpretation of the climatic record based on data reconstructions and correlations with key indicators. As such, the text is likely to spark debate and controversy at several levels. For example, many researchers today emphasize the differences of the warm climates during the Phanerozoic as opposed to the similarities. These workers are likely to view the modes as artificial distinctions or missing important climate information.

The Pliocene warm interval, for in-

stance, is the focus of international efforts to understand warm climates, yet it is characterized as part of a cold mode by the authors. The literature on the Early Eocene emphasizes a debate on whether this warm interval may be explained by a change in the role of ocean heat transport. Such a change would produce a climate very different from that of the Late Cretaceous. Yet the Late Cretaceous and the Early Eocene are described as part of one warm climate mode in the text.

The interpretation of particular climatic characteristics will also provoke debate. The authors describe the Late Cretaceous circulation as sluggish. Is this reasonable? Can we maintain warm poles with a weak atmospheric and oceanic circulation? The authors link the occurrence of evaporites to temperature. Is temperature alone a good predictor of the moisture balance? Does the restriction of seaways in the subtropics play a critical role? The authors introduce the possibility that the galactic year — the time taken by the Sun to orbit the centre of the Milky Way — may be correlated with the climate modes. Can this be tested and can we define a plausible mechanism that justifies this correlation?

As an interpretation of the climatic record, this book will prompt a lot of discussion because much of the interpretation is associated with uncertainty. But it is the most definitive reference so far on the interpretation of the climatic evolution of Earth as a series of alternating warm and cold modes. □

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Disruption of the CNTF gene results in motor neuron degeneration

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CNTF is a cytosolic molecule expressed postnatally in myelinating Schwann cells and in a subpopulation of astrocytes. Although CNTF administration prevents lesion-mediated and genetically determined motor neuron degeneration, its physiological function remained elusive. Here it is reported that abolition of CNTF gene expression by homologous recombination results in a progressive atrophy and loss of motor neurons in adult mice, which is functionally reflected by a small but significant reduction in muscle strength.

CILIARY neurotrophic factor (CNTF) has many activities *in vitro*^{1,2}, acting as a very potent survival factor for cultured parasympathetic¹, sympathetic¹, sensory¹ and spinal motor neurons^{2,4}, and hippocampal neurons⁵. Moreover, CNTF is a cholinergic differentiation factor (increasing choline acetyltransferase (ChAT) and reducing tyrosine hydroxylase (TH) levels) for primary cultures of rat sympathetic neurons⁶. It also promotes the survival of oligodendrocytes⁷ and the differentiation of O-2A glial precursor cells into type-II astrocytes *in vitro*⁸.

Furthermore, the local administration of CNTF *in vivo* prevents the degeneration of facial nerve motor neurons after axotomy in the early postnatal period⁹ and also markedly interferes with the degenerative changes in progressive motor neuronopathy (pmn) mutant mice¹⁰. Although these observations provide promising perspectives for the therapeutic use of CNTF, the physiological function of CNTF remains elusive. CNTF is expressed postnatally in myelinating Schwann cells and in a subpopulation of astrocytes¹¹. The structure of the molecule (lack of leader sequence)^{12,13}, its immunohistochemical localization in the cytosol^{11,14} and the absence of release of substantial quantities of CNTF into the culture medium of primary cultures of astrocytes¹⁵ and transfected Cos or HeLa cells^{12,13} characterize CNTF as a typical non-secreted cytosolic molecule. These characteristics strongly suggest that CNTF is not a physiological survival factor for motor neurons during embryonic development, because the period of naturally occurring motor neuron cell death in mice and rats is over at birth¹⁶. But the cytosolic localization of CNTF and the evidence against its secretion along the classic endoplasmic reticulum (ER)–Golgi pathway does not exclude the possibility of an unconventional, regulated release, for example, as suggested for basic fibroblast growth factor (FGF)^{17,18}. The localization of CNTF in myelinating Schwann cells and its relatively high quantities in the cytosol^{11,14,19} in comparison to its very high potency as a neurotrophic factor^{1,3} suggests that only a very small proportion of the cytosolic CNTF needs to reach the axons of the responsive neurons and that CNTF might have a maintenance function in postnatal neurons, in particular motor neurons. The site of synthesis and expected site of action of CNTF, together with its postnatal expression, invited an evaluation of its function by elimination of its expression by gene targeting.

Here we report on the abolition of CNTF expression by homologous recombination resulting in the disappearance both of CNTF protein and of the corresponding biological activity in the CNTF null (CNTF $-/-$) mutant mice. We concentrated on the evaluation of the physiological function of CNTF for motor neurons. As anticipated, the inactivation of CNTF expression did not affect the number of motor neurons during embryonic

development. Also during the first postnatal weeks neither functional nor morphological changes were apparent. But with increasing age, spinal motor neurons exhibited progressive atrophy and finally degeneration, functionally reflected by a small but statistically significant reduction in muscle strength.

Inactivation of the CNTF gene

The CNTF gene was disrupted according to established methods^{20,21}. The targeting vector used contained 9.5 kilobases (kb) of homologous CNTF genomic DNA in which the protein coding region of exon one was disrupted by insertion of a 1.1 kb MC1 *neo*^R expression cassette without a poly(A)⁺ signal. The *neo*^R protein coding sequence was followed by several translation stop codons to prevent any translational read-through (Fig. 1a).

D3 embryonic stem cells (2×10^7) (ref. 22) were transfected with the linearized targeting vector by electroporation. The cells were selected in medium containing $380 \mu\text{g ml}^{-1}$ G418 (positive selection) and $2 \mu\text{M}$ of gancyclovir (negative selection). Of the 690 G418-resistant clones initially screened by polymerase chain reaction (PCR), 4 had the expected 0.6 kb DNA fragment with a frequency of 1 in 170 resistant clones. The results obtained by the PCR procedures were verified by Southern blot analysis in the corresponding clones.

TABLE 1 Number of facial motor neurons in controls and CNTF $-/-$ mice

Age	CNTF $+/+$ and $+/-$	CNTF $-/-$
4 weeks	1,998 $\pm$ 131 (n=6)	2,033 $\pm$ 90 (n=7)
28 weeks	2,092 $\pm$ 106 (n=7)	1,620 $\pm$ 73* (n=8)

The brain stems from 4- and 28-week-old CNTF $-/-$ mice were perfused with 4% formaldehyde and prepared for paraffin serial sections ($7 \mu\text{m}$) as described^{9,10}. Briefly, the sections were Nissl-stained, and nucleoli of facial motor neurons were determined in every fifth section. Age-matched CNTF $+/+$ and $+/-$ mice (whose levels of CNTF protein and bioactivity were indistinguishable in sciatic nerve extracts (data not shown) were used as controls. The diameters of the nucleoli were measured at high magnification ($\times 1,000$). Average diameter of nucleoli in each group: 4-week-old $+/+$ and $+/-$ mice, $3.7 \pm 0.7 \mu\text{m}$ (mean $\pm$ s.d. for 54 determinations); 4-week-old $-/-$ mice, $3.6 \pm 0.4 \mu\text{m}$ (mean $\pm$ s.d. from 55 determinations); 28-week-old $+/+$ and $+/-$ mice, $3.3 \pm 0.4 \mu\text{m}$ (mean $\pm$ s.d. from 54 determinations); 28-week-old $-/-$ mice, $3.5 \pm 0.4 \mu\text{m}$ (mean $\pm$ s.d. from 52 determinations). The counts of facial nucleoli of each animal (mean from independently counted left and right side) were corrected according to ref. 33. Values shown are mean $\pm$ s.e.m. of each group. Statistical significance of the difference between (corrected) motor neuron number in 28-week-old CNTF $-/-$ mice and controls was tested by Student's t-test.

* The two groups are different at the 0.005 level ($P=0.002$).

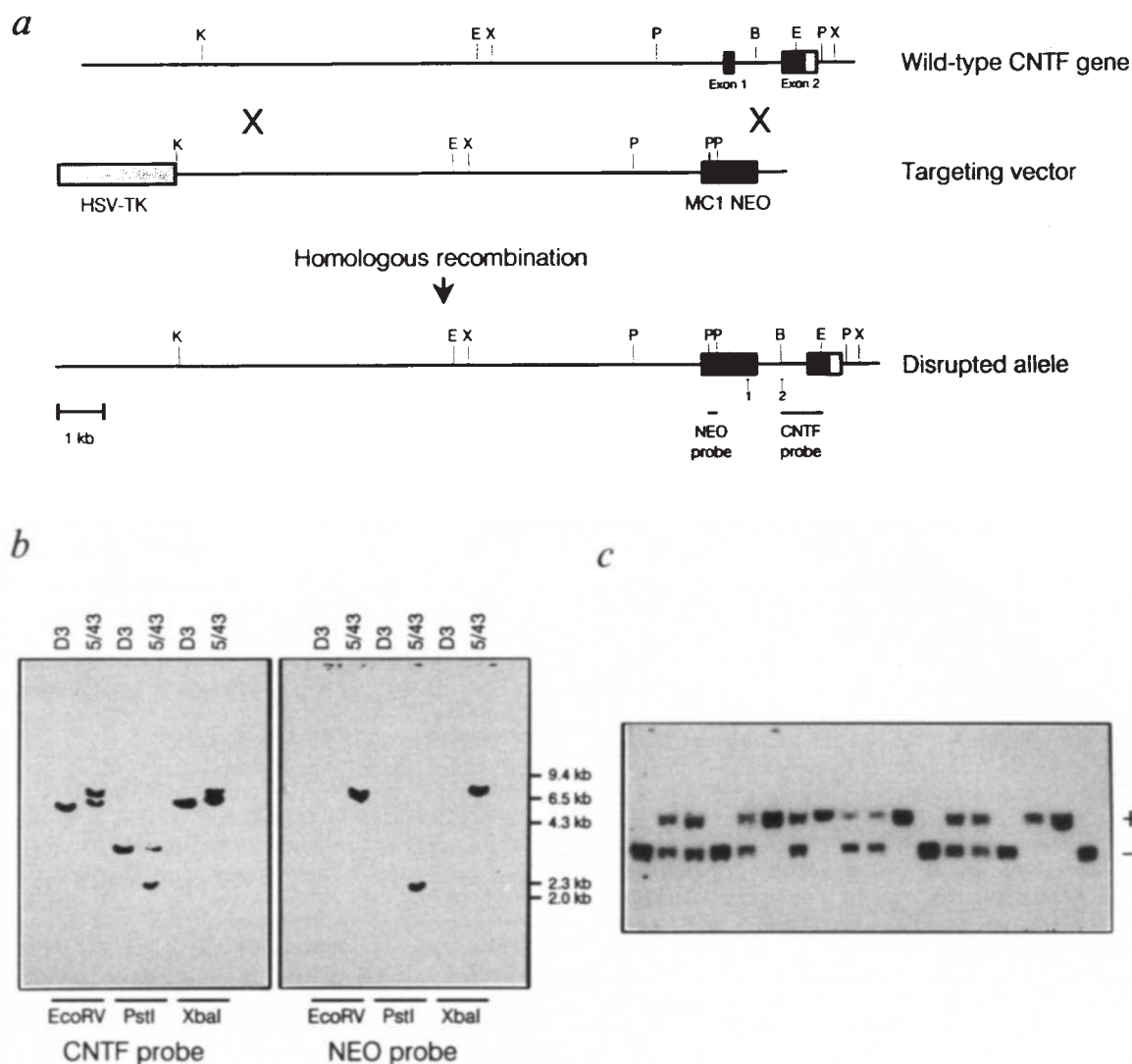


FIG. 1 Disruption of the CNTF gene in mouse embryonic stem (ES) cells. **a**, Targeting vector. Top line, Normal genomic structure of the mouse CNTF gene. Exons 1 and 2 are shown as boxes, black boxes represent coding regions. Middle line, Targeting replacement vector used to disrupt the endogenous CNTF gene. The MC1 neo-cassette lacking a polyadenylation signal was inserted into the *NheI* site 63 base pairs (bp) downstream of the initiator codon. Homologous recombination will generate a fusion gene of neomycin and the endogenous CNTF 3' untranslated region. Negative selection using gancyclovir against HSV thymidine kinase gene expression did not lead to an enrichment of targeted clones in our experiment. PCR primers 1 (5'-CCTTCTATCGCCTTCTTGAC) and 2 (5'-AAACAAGCCCAGAACTGTGG) were used to identify targeting events in *neo*^R ES cells. The bottom line represents the structure of the mutated CNTF gene. Restriction enzyme abbreviations: K, *KpnI*; E, *EcoRV*; X, *XbaI*; P, *PstI*; B, *BglII*. **b**, Southern blot analysis of ES cell clone 5/43 containing a targeted disruption of the CNTF gene. DNA samples from D3 ES cell clone 5/43 were digested with *EcoRV*, *PstI* or *XbaI* and analysed with two different probes. Left, CNTF probe, 850 bp *BglII*-*EcoRV* fragment of the CNTF gene not present in the targeting vector DNA; right, Neo probe, 200 bp *PstI* fragment of the *neo* gene. Molecular size markers are shown to the right. **c**, Southern blot analysis of DNA from offspring from CNTF ^{+/}- x CNTF ^{+/}- mating. DNA was extracted from tail-tips, digested with *PstI* and analysed with the CNTF probe as described in **b**. The mutated CNTF gene resulted in a shorter fragment as shown in **b**. The position of the wild-type and mutated alleles are indicated on the right.

METHODS. **a**, The CNTF targeting vector was constructed by subcloning a 9.5 kb *KpnI*-*BglII* fragment containing exon 1 of murine genomic CNTF DNA into pBluescript KS (Stratagene). A 1.1 kb *SaII*-*XhoI* fragment of

the MC1 *neo* cassette (without a poly(A)⁺ signal) was inserted into the *NheI* site which was converted to a *XhoI* site. An HSV-TK cassette was then inserted at the *KpnI* site. D3 ES cells were cultured (in DMEM with 15% FCS and 60% Buffalo rat liver cell conditioned Glasgow medium) on embryonic fibroblast feeder layers derived from neomycin-resistant transgenic mice. ES cells (2×10^7) in PBS, divided equally into five cuvettes, were electroporated with 125 μ g *NotI*-linearized targeting vector DNA at 240 V, <500 μ F (Biorad Gene Pulser). Cells were selected in medium containing 380 μ g ml⁻¹ G418 and 2 μ M gancyclovir for 10–14 days. The resistant clones were picked and placed into 48-well plates with feeder layers. Cells were trypsinized the next day, and a 40-cycle PCR amplification (40 s at 94°C, 1 min at 55°C and 3 min at 70°C) was performed on pools (each containing about 10^4 cells) of 10 clones to screen for homologous recombinant clones. PCR primers 1 and 2 would be expected to give rise to a 600 bp product only when the vector was integrated correctly into the CNTF locus. Individual targeted clones from positive pools were identified by PCR (data not shown). Correct homologous recombination was confirmed by Southern blot analysis. **b**, The positive ES cell clone (5/43) and D3 ES cells were expanded and DNA isolated³⁶. DNA (10 μ g) was digested with restriction enzymes as indicated, electrophoresed in 0.6% agarose gels in 1 $\times$ TBE (89 mM Tris borate, 1 mM EDTA) buffer, and blotted under alkali conditions to Hybond N+ (Amersham) membranes. Prehybridization was done for 3 h at 65°C in 1 M NaCl, 1% SDS, 1 mM EDTA and 10 mM Tris-HCl containing 10% dextran sulphate. Hybridization was in the same buffer using ³²P-labelled probe (10×10^6 c.p.m. ml⁻¹). The membranes were washed for 10 min at room temperature in 2 $\times$ SSC, twice for 30 min at 65°C in 0.1 $\times$ SSC, 1% SDS and then exposed to X-ray film (Kodak X-Omat).

For blastocyst injection we selected clone 5/43 which showed correct integration of the targeting vector at the CNTF locus (Southern blot analysis, Fig. 1b). Moreover, this clone showed a virtually exclusive diploid (40 chromosomes) chromosomal pattern (data not shown). Chimaeric mice were generated by microinjection of 5/43 cells into 3.5-day-old blastocysts from C57BL/6 mice (non-agouti). As the D3 embryonic stem (ES) cell line is derived from the 129/SV strain (agouti), chimaeric mice were identified by coat colour. The ES cell contribution as estimated by agouti pigmentation averaged 80% for the whole experimental series. Male chimaeric mice were tested for germ-line transmission of the mutated CNTF gene. From 12 males analysed, 2 showed germ-line transmission as demonstrated by the production of agouti offspring (for details, see ref. 23). CNTF $+/-$ heterozygotes were mated and 98 offspring were evaluated by Southern blot analysis. The ratio of CNTF $+/+$, $+/-$ and $-/-$ in the offspring was about 1:2:1 following mendelian rules (Fig. 1c).

Effectiveness of gene targeting

Western blot analysis of sciatic nerve extracts demonstrated that the immunoreactivity detected in control mice by an IgG fraction of rabbit anti-CNTF antiserum¹⁹ was absent in CNTF $-/-$ mice (Fig. 2a). Accordingly, no CNTF-assignable survival activity was present in the corresponding sciatic nerve extracts, as evaluated with embryonic chick ciliary neurons in culture (Fig. 2b). Even in the presence of 1,000 ng protein from sciatic nerve extracts of CNTF $-/-$ mice, no survival of ciliary neurons was detectable, whereas 100 ng sciatic protein extract from CNTF $+/+$ mice already showed maximal responses. Moreover, the ciliary neuronal survival activity of CNTF $+/+$ sciatic nerves could be completely blocked by an IgG fraction of a blocking CNTF antiserum. Thus the protein product of the CNTF gene was absent in CNTF $-/-$ mice as expected from the genomic findings.

CNTF inactivation on spinal cord motor neurons

The morphometric analysis of lumbar spinal cord motor neurons (Fig. 3a) demonstrated no detectable difference between 4-week-old CNTF $-/-$ and CNTF $+/+$ mice. Moreover, the number of facial motor neurons was not significantly different ($P=0.8$), as shown by cell counting in serial sections of the brain stem (Table 1). This indicates that the absence of CNTF protein does not influence the development of motor neurons during the first postnatal weeks. But in 8-week-old animals, lumbar motor neuron cell bodies were smaller, and the nuclei swollen (Fig. 3c) resulting in an average increase in the ratio between nuclear and cytoplasmic areas of about 40% (Fig. 3a). Both the differences between cell sizes and the ratios of nuclear and cytoplasmic areas were statistically significant ($P<0.001$). In 14-week-old animals, both the lumbar motor neuron cell area and the nuclear area were smaller in CNTF $-/-$ mice than in CNTF $+/+$ mice, resulting in an apparent 'normalization' of the ratio between the nuclear and cytoplasmic areas of these cells (Fig. 3a).

In CNTF $-/-$ mice some motor neurons showed a distinct reduction of the Nissl structure (Fig. 3c), and others were surrounded by cells with small nuclei resembling activated glial cells (Fig. 3d, e). Similar morphological changes became apparent in sections of facial nuclei, which were prepared by different fixation, embedding and staining procedures (Fig. 3h-k). These morphological changes were already detectable in 8-week-old CNTF $-/-$ animals (Fig. 3d) and became more pronounced in 24-week-old animals (Fig. 3e, f) compared to controls (Fig. 3g). In 28-week-old animals the number of facial motor neurons was reduced by 22% compared to age-matched controls ($P=0.002$) (Table 1), demonstrating the progression of the atrophic to degenerative changes, resulting finally in motor neuron cell death. The facial nucleus was chosen for counting motor neuron cell numbers as this clearly delineated structure in the brain stem does not contain interneurons, and therefore permits a more

reliable quantification than the analysis of the ventrolateral column of the spinal cord.

In parallel with the degenerative changes and loss of motor neurons, there was a small but statistically significant ($P=0.034$) reduction in muscle strength in 28-week-old animals (Table 2). The progressive atrophic and degenerative changes in lumbar spinal motor neurons were accompanied by an increased number

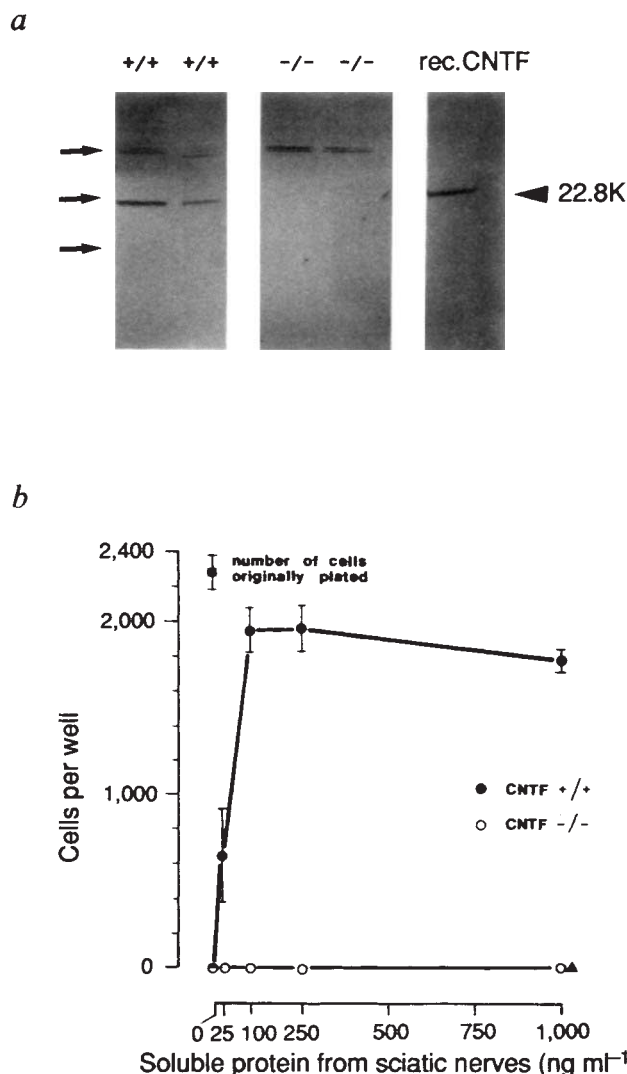


FIG. 2 Analysis of CNTF protein expression and biological activity in CNTF $-/-$ mice. **a**, Western blot analysis of sciatic nerve extracts. Sciatic nerves from adult CNTF $+/+$ and CNTF $-/-$ mice were prepared, homogenized and ultracentrifuged as described¹⁹. Protein (30 μ g per lane) was electrophoresed. The arrows at the left indicate the position of molecular mass markers (ovalbumin, 45K; trypsinogen, 24K; lysozyme, 14.3K). CNTF immunoreactive bands were detected by a rabbit antiserum (1:1,000) against rat CNTF. CNTF immunoreactivity is absent in extracts from CNTF $-/-$ mice whereas a clear band of the expected size (22.8K) is detectable in CNTF $+/+$ nerve extracts. The doublet band of about 44K was also recognized in the absence of the primary antiserum and most probably represents immunoglobulin heavy chains recognized by the secondary antiserum. **b**, CNTF-bioactivity in nerve extracts from CNTF $+/+$ and CNTF $-/-$ mice. Embryonic (day 8) chick ciliary neurons were prepared and cultured (for 24 h) as described¹⁹. Protein extracts from sciatic nerves were added at the concentrations shown. Each determination was done at least in duplicates. The survival activity present in 1 mg of nerve extracts from CNTF $+/+$ mice could be completely blocked by the addition of 6 μ g ml⁻¹ of an IgG fraction of a blocking CNTF antiserum¹⁰, demonstrating that the survival activity of sciatic nerve extracts in this assay stems exclusively from CNTF.

of activated microglial cells (Fig. 4), as visualized by horseradish peroxidase conjugates of the B4 isolectin from *Griffonia simplicifolia*^{24,25}. The ventrolateral region of the spinal cord of CNTF $-/-$ mice contained more lectin-labelled microglial cells in comparison to the corresponding regions of CNTF $+/+$ mice.

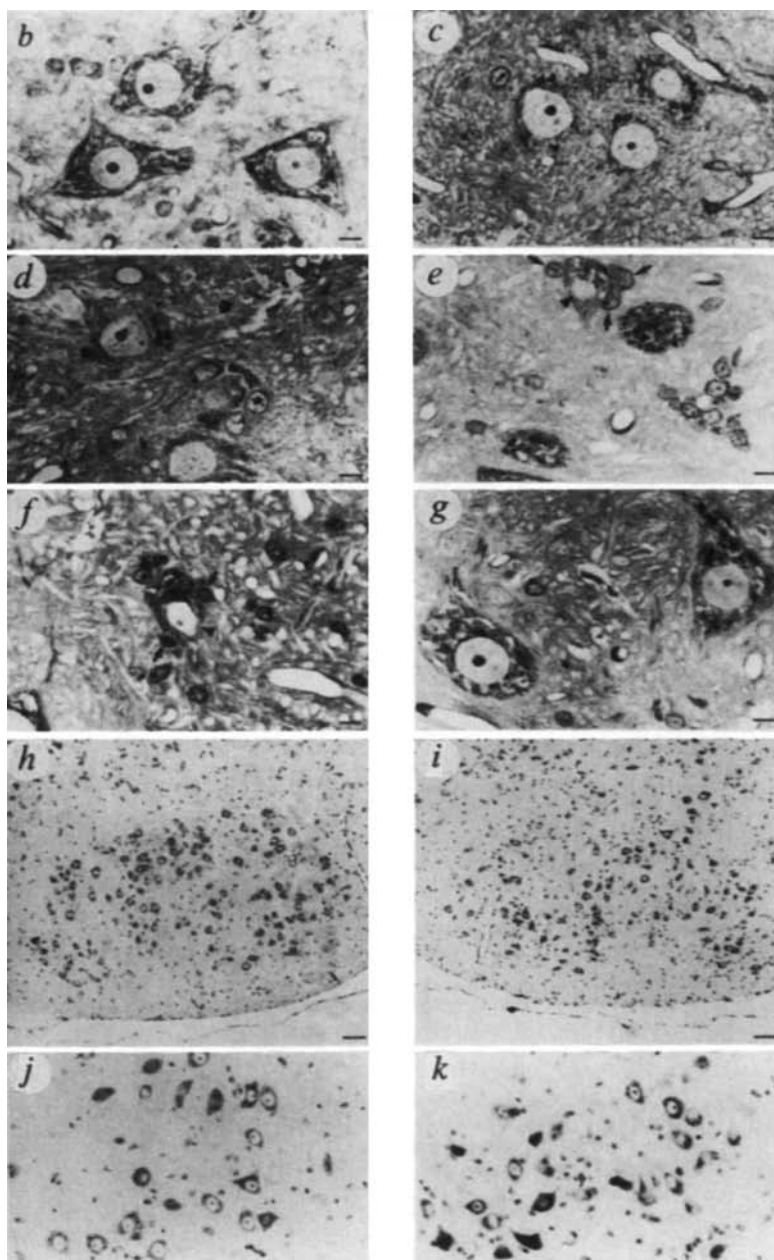
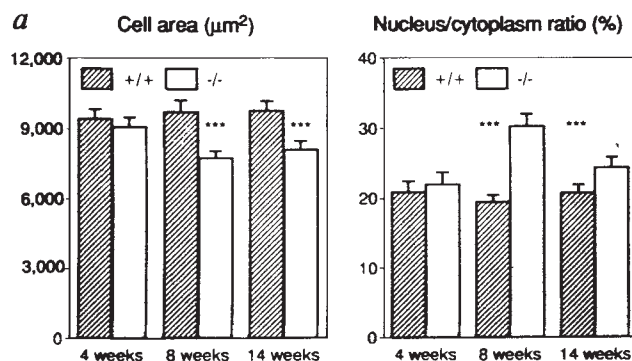


FIG. 3 Analysis of motor neuron cell size and structure in CNTF $-/-$ mice. **a**, Morphometry of lumbar motor neuron cell size and determination of the nucleus/cell ratio. **b**, Motor neurons in CNTF $+/+$ mice (8 weeks old). **c**, Motor neurons in CNTF $-/-$ mice (8 weeks old). **d**, **e**, **f**, Ventrolateral spinal cord region of 8-week-old (**d**) and 24-week-old (**e**, **f**) CNTF $-/-$ mice showing motor neurons with reactive glial cells (arrows) and degenerating neurons (arrowhead). **g**, Lumbar motor neurons from a 24-week-old CNTF $+/+$ control mouse. **h**–**k**, Facial motor neurons in 6-month-old CNTF $+/+$ (**h**, **j**) and $-/-$ mice (**i**, **k**) at low (**h**, **i**) and high (**j**, **k**) magnification. Brain stems were prepared for Nissl stained paraffin sections as described in legend to Table 1. Scale bars in **b**–**g**, **j**, **k**, 10 μm; in **h**, **i**, 50 μm.

METHODS. CNTF $-/-$ and CNTF $+/+$ mice of different ages were perfused first with PBS containing 0.002% butanilicain and then with 4% paraformaldehyde/1% glutaraldehyde. The lumbar spinal cords (level L3–L5) were dissected and postfixed with 2% glutaraldehyde in a sodium-cacodylate buffer for at least three days at 4 °C (ref. 37). After rinsing in PBS containing 3.4% sucrose, the tissues were incubated with osmium tetroxide (1%) in a 2% potassium dichromate solution and processed for Epon embedding according to standard procedures³⁸. Semi-thin sections (1 μm) were prepared and stained with toluidine blue. Sections were analysed with a Zeiss Axiophot microscope (magnification $\times 1,000$) and morphometry was done using a Bioscan Optimas picture analysis hard- and software coupled to the microscope. Total cell and nuclear areas from motor neurons with typical Nissl structure and a clearly identifiable nucleolus were analysed from 10 sections per animal. These sections were at least 10 μm apart from each other to avoid double analysis of the same cells. At least 160 motor neurons were analysed for each age group in CNTF $+/+$ animals, and more than 210 motor neurons were analysed for each age group in CNTF $-/-$ mice. Statistical analysis. Analysis of variance was done on cell and nuclear area data from CNTF $+/+$ and CNTF $-/-$ mice taking into account variances due to sections and individual animals. The General Linear Models (GLM) procedure (SAS Institute Inc.)³⁵ was used to calculate least squares means (LSM) and standard errors (s.e.). LSM were compared within age class using Student's *t*-test. Data shown represent LSM and s.e. for cell area and nuclear area/cytoplasmic area ratio of alpha motor neurons from CNTF $+/+$ and CNTF $-/-$ mice at different ages as indicated. *** Statistically significant differences ($P < 0.001$).

Although the intensity of the microglial staining in CNTF $-/-$ mice varied between experiments (Fig. 4d, f), there was a distinct difference compared to CNTF $+/+$ mice in all experiments. In addition to the increase in lectin binding, a significant proportion of the microglial cells in spinal cords of CNTF $-/-$ mice were closely associated with motor neurons and showed a reduced branching of their cytoplasmic processes, exhibiting features of activated microglia²⁵ (Fig. 4b, d, f).

Discussion

These investigations evaluated whether CNTF is involved in the physiological maintenance of structure and function of motor neurons. It was already known that CNTF is a cytosolic molecule whose expression is restricted to myelinating Schwann cells and a subpopulation of astrocytes^{11,14}. The restricted expression of CNTF both in the periphery and in the CNS is in distinct contrast to the very broad spectrum of its biological actions *in vitro*^{1,8}. Among these, the effective support of motor neurons *in vitro* and *in vivo* was of particular interest. The levels of CNTF protein in the sciatic nerve are about 10,000 times

higher than those of the secretory nerve growth factor (NGF)¹⁹. But because of its cytosolic location, the question arose whether CNTF becomes available to the responsive neurons under normal conditions and whether therefore CNTF has a physiological function at all. The quantities of CNTF released into the medium by primary cultures of Schwann cells and astrocytes are very small and are within the limits of the release of cytosolic molecules by normal cell death under the given culture conditions¹⁵. Therefore, it would be difficult to detect additional small quantities of CNTF released by a non-conventional, regulated mechanism as has been demonstrated, for example for basic FGF^{17,18}. Moreover, even if such a mechanism could be identified and characterized *in vitro*, would the observations made be representative for the situation *in vivo* where Schwann cells express much higher CNTF levels than Schwann cells in culture and, as myelinating cells, are in a distinctly different structural and functional situation? Thus, it was essential to evaluate the potential physiological function of CNTF *in vivo*. Because the spatial relationship between the CNTF-producing Schwann cells and the responsive neurons precluded the use of antibodies, the abolition of CNTF production by homologous recombination was the chosen method. The structure and function of motor neurons in the early postnatal phase would be expected to be normal

given that the expression of CNTF starts in the first postnatal week and does not reach adult levels before the 4th week^{2,13}. Indeed, no changes in the morphology or function were detectable in 4-week-old animals. But subsequently there was a gradually increasing atrophy of spinal motor neurons which first became apparent at postnatal week 8. Furthermore, in the 28-week-old animals, a statistically ($P=0.002$) significant reduction by 22% in motor neuron cell numbers became apparent (Table 1) in the well delineated facial nucleus. The slowly progressing atrophy and final degeneration of motor neurons was accompanied by an increasing number of reactive microglial cells in the vicinity of the motor neurons. In contrast, increased GFAP staining in reactive astrocytes, which is a characteristic of acutely degenerating motor neurons²⁶, could not be observed. This is reminiscent of the absence of enhanced GFAP reactivity in the genetically determined progressive degeneration of motor neurons in dogs²⁷. Even at 22 weeks, when the first signs of motor neuron degeneration became apparent, there was still no evidence of an impaired motor neuron function (Table 2). But at 28 weeks a small but statistically significant ($P=0.034$) reduction in muscle strength (Table 2) became apparent. Major differences were, in any case, not expected because in many neuronal systems a high proportion of neurons have to degenerate before

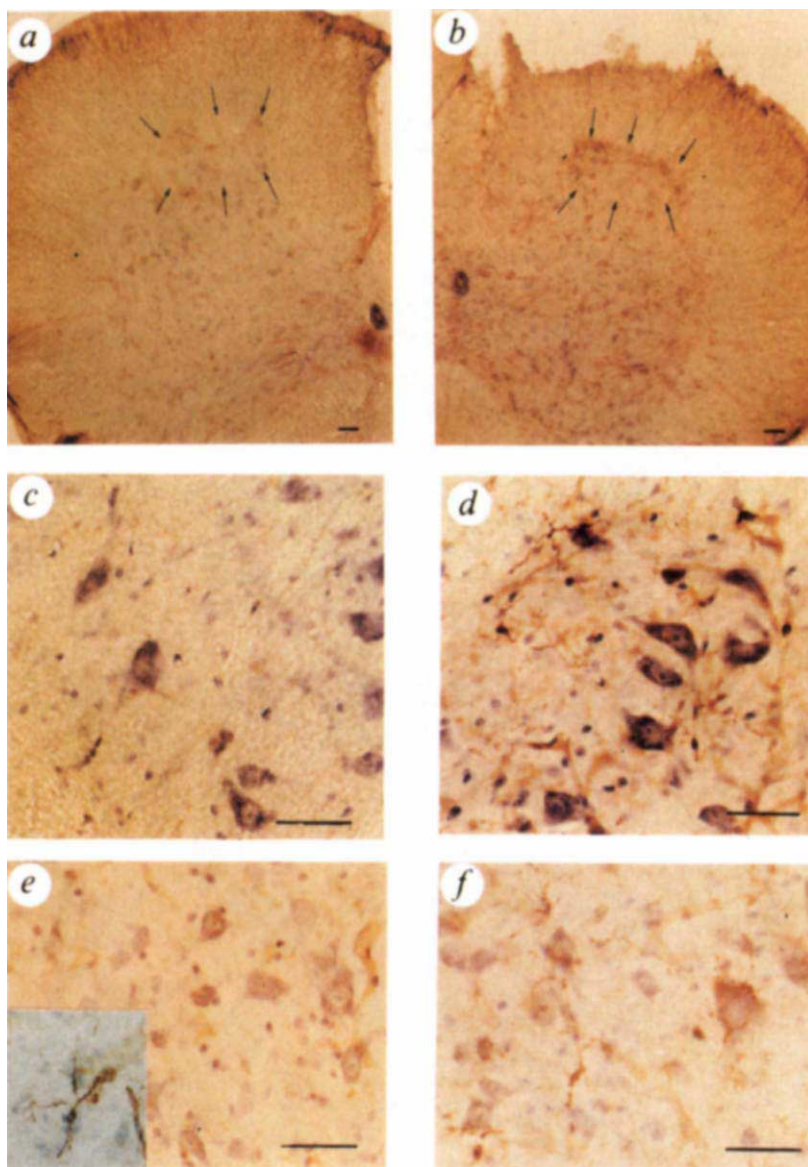


FIG. 4 Microglia reaction in the anterior horn of the upper lumbar spinal cord of CNTF $-/-$ mice. a, b, e, f, 24-week-old CNTF $+/+$ mice (a, e) and CNTF $-/-$ mice (b, f) at low (a, b) and high magnification (e, f). The anterior horn region of the spinal cord in a and b is arrowed. Insert in e shows the appearance of a microglial cell within the anterior horn region from the same CNTF $+/+$ animal. c, d, 8-week-old control (CNTF $+/+$) (c) and CNTF $-/-$ mouse (d). Each pair of figures (a/b, c/d, e/f) was derived from independent experiments and the processing for each pair of sections was identical. After preparation of vibratome sections (40 μ m) of paraformaldehyde (4%) fixed upper lumbar spinal cords, microglia was identified by lectin binding (Griffonia simplicifolia (GSA-B4) coupled to horseradish peroxidase (Sigma, L539)) as described²⁴. Diaminobenzidine was used to visualize microglia, sections were counterstained with cresyl violet. The specificity of the lectin Griffonia simplicifolia for microglia is not absolute, it also stains endothelial cells and basal membranes. Scale bars, 50 μ m.

TABLE 2 Forelimb grip strength of male CNTF $+/+$ and CNTF $-/-$ mice at different ages

Age	n	Mean grip strength (in Newtons)		n		P
		CNTF $+/+$	CNTF $-/-$			
8 Weeks	7	1.149 $\pm$ 0.013	3	1.117 $\pm$ 0.020		$P=0.187$
22 Weeks	10	1.288 $\pm$ 0.012	8	1.314 $\pm$ 0.013		$P=0.131$
28 Weeks	5	1.203 $\pm$ 0.040	13	1.107 $\pm$ 0.020		$P=0.034$

Forelimb grip strength (in Newtons) was determined in male CNTF $+/+$ and $-/-$ mice using the automated grip strength meter described in ref. 34. Briefly, after the animals were placed on a platform and allowed to grasp a triangular ring, they were pulled away until the grip was broken. Grip strength was determined with a computerized electronic pull strain gauge (Columbus Instruments) which was directly fitted to the grasping ring. Seven measurements were taken from each animal within a minute. The grip strength was measured blindly, the person measuring the strength did not know whether the mice were CNTF $+/+$ or $-/-$. Data were analysed by analysis of variance taking into account the variation of data within each animal. The GLM procedure (SAS Institute, Inc.) was used to calculate the least-squares means (LSM) and standard errors (s.e.)³⁵ shown for each group. Student's *t*-test was used to compare LSM of CNTF $+/+$ and CNTF $-/-$ mice within the same age class. Significance of differences (*P* values) between groups are shown in the last column.

signs of functional deficiency become apparent. For example, in patients suffering from amyotrophic lateral sclerosis, accumulating evidence suggests that the first clinical symptoms usually do not occur before about 50% of the spinal motor neurons are lost²⁸. This would agree with the situation in CNTF $-/-$ mice. Future experiments with older CNTF $-/-$ animals will show whether the absence of the CNTF supporting function will lead to further degenerative changes and further reduction in muscle strength leading finally to a clearly detectable impairment of gross motor neuron function. In this context, it will be particularly interesting to see whether compensatory mechanisms come into play, for example the reactive production of BDNF by Schwann cells, provided a sufficiently high level of axon degeneration has been reached, and whether a similar reactive production of BDNF would be obtained as after nerve lesion²⁹.

The targeting experiments show that CNTF plays an essential role in the maintenance of motor neuron function in the postnatal period. But they do not reveal how CNTF is transferred from Schwann cells to its motor neuron targets. This might occur via regulated membrane transfer by a specific unconventional mechanism or after uncontrolled release from the cytosol resulting from incidental discontinuity of the plasma membrane. The

microtrauma that nerves are continuously subjected to by normal mechanical stress could essentially contribute to the latter mechanism. For instance, in rat skin horseradish peroxidase injected into the dermal stroma penetrates into epidermal epithelial cells in consequence of physical activity, resulting in transient membrane wounding permitting the penetration of this molecule, which is larger than CNTF³⁰. In either case, the quantities of CNTF required by the responsive neurons need to be only extremely small.

Other possible physiological functions of CNTF are expected to become apparent during postnatal development only, although many *in vitro* effects have been observed in embryonic tissues. *In vitro* effects of CNTF may be accomplished *in vivo* by other mechanisms and may gain relative importance when the expression of CNTF is abolished. This seems to be the case for the induction of cholinergic properties in adrenergic sympathetic neurons by CNTF, which are characterized by an increase in ChAT and vasoactive intestinal peptide (VIP) levels and a reduction in TH^{6,31}. In preliminary experiments (data not shown), we compared the innervation of sweat glands of CNTF $+/+$ and CNTF $-/-$ mice using immunohistochemical methods and could not find any difference in VIP positive nerve fibres innervating these structures within the footpads. Also ChAT levels of the footpad extracts were not altered in CNTF $-/-$ mice. If CNTF plays a role as a cholinergic differentiation factor, it is certainly not the only molecule responsible for the induction of cholinergic properties in sympathetic neurons innervating the sweat glands. Leukaemia inhibitory factor (LIF) null mutant mice also showed a normal VIP innervation of the sweat glands in the footpads³². Besides a broad spectrum of action on the haematopoietic and lymphatic system, LIF has similar effects as CNTF on cultured sympathetic neurons increasing ChAT and reducing TH activity. It will be of interest to see whether crossbreeding of LIF and CNTF null mutants (experiments that are in progress) will abolish the transformation of sympathetic adrenergic neurons into cholinergic ones by the sweat glands or whether *in vivo* additional, not yet identified, molecules are responsible for the changes in the properties of sympathetic neurons.

In conclusion, the elimination of the CNTF gene by homologous recombination has demonstrated that the expression of this gene is not necessary for the development of spinal motor neurons as assessed by morphological criteria. But this approach has also shown that CNTF has an essential maintenance function for motor neurons in the postnatal period. To determine other potential physiological functions of CNTF, we expect that similar, very detailed analyses will be necessary to identify subtle, gradually appearing changes in the postnatal phase. □

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Identification of a supernova remnant coincident with the soft γ -ray repeater SGR1806–20

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THE 'soft' spectra, short duration and recurrent activity of the three known soft γ -ray repeaters (SGRs) distinguishes them from the majority of cosmic γ -ray bursts^{1–4}. Although there is no generally accepted model for the repeaters, it has been argued⁵ that they are associated with radio pulsars: this is consistent with the coincidence⁶ of SGR0526–66 with the supernova remnant N49 in the Large Magellanic Cloud, and the location of the two other repeaters at low galactic latitudes. Here we show that the well localized repeater SGR1806–20 (refs 2, 3) is also coincident with a supernova remnant: the amorphous radio nebula G10.0–0.3 (refs 7, 8). Taken together, these two associations argue strongly in favour of a neutron-star origin for the repeaters. We suggest that all young pulsars—that is, neutron stars still embedded in their supernova remnants—pass through a brief (~ 500 year) phase of SGR activity. The detection of pulsar-powered synchrotron nebulae in, or pulsations from, the two supernova remnants coincident with SGRs would confirm this model.

SGR0526–66, with a localization error of 0.1 arcmin², is coincident with⁶ the supernova remnant (SNR) N49 in the Large Magellanic Cloud (LMC). The chance-coincidence probability is small⁹, $\lesssim 10^{-3}$. Excluding the one anomalously bright burst of 5 March 1979 (identified with SGR0526–66), the ratios of the peak burst fluxes for the three SGRs are consistent with the location of SGR0526–66 in the LMC (distance, $d \sim 55$ kpc) and roughly Galactocentric distances ($d \sim 10$ kpc) for the remaining two^{5,10}. The exclusion of the anomalous burst is justified⁵ as it had a peak brightness $\sim 10^3$ times that of typical bursts, strongly suggesting that it originates from an entirely different mechanism. This SNR–SGR association has been questioned on theoretical grounds because of the implied high luminosity during the anomalous burst (see refs 10, 11 for reviews). In our opinion, however, the circumstantial evidence in favour of the association is compelling. The logical next step is to determine if the remaining SGRs are also associated with SNRs.

SGR1806–20, with a localization function³ of 450 arcmin², has the second-best localization and is therefore suitable for further investigations. A search of Green's SNR catalogue⁸ showed that the supernova remnant G10.0–0.3, a 6-arcmin radio nebula, is contained within the localization function. This object, first studied with the Molonglo (408 MHz) and Parkes (5 GHz) radio telescopes, was found to have a negative spectral index, $\alpha = -0.8$; here flux at frequency ν , $S_\nu \propto \nu^\alpha$, and the negative value of α is characteristic of a non-thermal emission. It was classified⁷ as an SNR because it is an extended source at low Galactic latitude with a non-thermal spectrum. It is clearly seen in the Molonglo image¹² but two conflicting estimates of its flux (S) are tabulated: 1.9 Jy (ref. 12) and 5.4 Jy (ref. 7). Inspection of the image suggests a value between the two, $S \sim 3$ Jy. The object is not detectable in the 2.7-GHz survey¹³ using the Bonn 100-m radio telescope, showing that the flux at this frequency $S \lesssim 0.2$ Jy. So we confirm that the spectrum is non-thermal.

Little else is known about G10.0–0.3. Fortunately, three

datasets from the Very Large Array (VLA), one at 330 MHz (resolution 50 arcsec; Fig. 1) and two at 1.4 GHz (both archival, one at 'high' resolution, 10 arcsec; and the other at 'low' resolution, 50 arcsec; Fig. 2), were available.

The morphology of G10.0–0.3 is not that of a typical young SNR: neither a centre-brightened nor an edge-brightened shell. In the former case, the central emission region (the 'plerion') is due to a synchrotron nebula powered by a pulsar: the latter is a blast-wave-driven SNR. The irregular morphology, with two components, is reminiscent of an evolved SNR although the possibility exists that these are two unrelated objects. The curved southern feature has the appearance of an SNR shell. The northern feature can be interpreted conceivably as a core-dominated triple radio galaxy (for example, ref. 14). But the high resolution 1.4 GHz dataset did not reveal any point nuclear emission, implying that $S \lesssim 6$ mJy (3σ), inconsistent with the extragalactic hypothesis. No spatial structure is seen in the image, consistent with both components being diffuse nebulae. We conclude that G10.0–0.3 is an amorphous, non-thermal source and most probably an evolved SNR.

If the SNR classification is correct, a distance estimate may be obtained using the surface-brightness/diameter relation (for

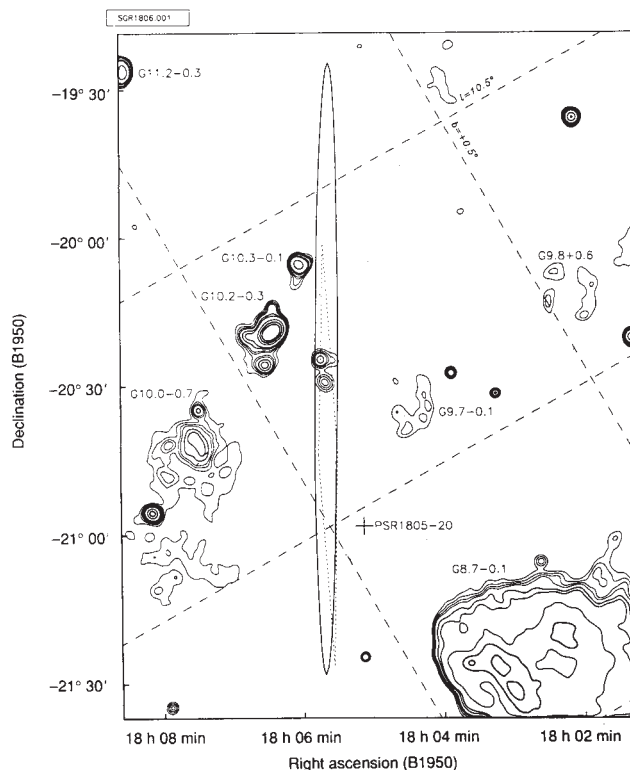


FIG. 1 Radio image of the region around G10.0–0.3 (which is itself within the vertical ellipse) obtained with the VLA at 330 MHz for another project (D.A.F., N. Kassim and K. Weiler, manuscript in preparation). Total integration time is 240 min, but G10.0–0.3 is 81 arcmin away from the pointing centre and the half-power radius of the image is only 70 arcmin. The original image has been smoothed from its original resolution of 50 arcsec to 2 arcmin. Contour intervals are 4, 6, 8, 10, 15, 20, 40, 60, and 80 times the r.m.s. noise level of 12 mJy beam⁻¹. There are five SNRs in this field, several H II regions and one known pulsar PSR1805–20. According to ref. 3 SGR1806–20 is localized to an ellipse with major axis of 2.06° (declination) and 4.4 arcmin (right ascension) centred on $\alpha(1950) = 18^h 05^m 38^s$ and $\delta(1950) = -20^\circ 26' 40''$. The ellipse appears to be remarkably centred on the SNR and there are no other radio sources within the bounds of the ellipse. The diamond-shaped region (dashed line) is a new localization obtained by J.-L. Atteia (personal communication).

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example, ref. 15); this gives $d \sim 17$ kpc and an inferred age of 5×10^3 yr. But this relation is valid only if supernovae form an homogenous class and expand into similar media; neither is true. For this reason, we view such estimates with some suspicion.

Given the non-negligible size of the localization function it is important to assess the probability of chance coincidence. In deriving such *a posteriori* assessments, it is important to state the assumptions clearly. Our null hypothesis is that SGRs are unrelated to SNRs and are isotropically distributed on the sky. The probability, p , that an SGR localization will coincide with an unrelated SNR is then calculated. The restriction of the discussion to the class of SNRs is suggested by the N49/SGR0526–66 association. If the estimated p is large, any apparent coincidence of SGR1806–20 with an SNR cannot be taken seriously. By contrast, a small p is consistent with our model that SGRs are associated with SNRs. Note that owing to the simplified model, a small value of p does not prove the hypothesis.

The calculation of p is complicated by the unusual shape of the localization function, an ellipse with major axis $\delta_1 = 2.06^\circ$ and minor axis $\alpha_1 = 4.4$ arcmin. Consider the equivalent problem of randomly shooting a bullet, whose cross-section is that of the localization function, at a map with islands of arbitrary shape (corresponding to our SNRs). A shot is considered to be a hit if any part of the bullet intersects at least one island. For the purpose of evaluating p , the effective 'hit' area of an island, a ,

is that enclosed by the convolution of the shape of the island with the shape of the bullet. Thus $p = \sum a/C$ where the summation is over the islands and C is the total area of the map.

In our specific case, $C = 4\pi$ and

$$p \sim \frac{1}{4\pi} \sum_j \max(\alpha_1, \Delta\alpha(j)) \max(\delta_1, \Delta\delta(j)) \quad (1)$$

where $\Delta\alpha(j)$, $\Delta\delta(j)$ is the angular size of the j th SNR, obtained from ref. 8. We find $p \sim 5 \times 10^{-3}$. Two factors make p an upper limit. First, in the inner Galaxy (where most of the SNRs are present) the SNR density is high enough that, given the large δ_1 , the new boundaries (after convolution) may overlap. Second, a few very old, nearby and hence large diameter SNRs are included in the SNR catalogue. Such remnants will not be detectable at typical SNR distances and should not be counted. After excluding the nine largest diameter remnants and counting only the larger of pairs of remnants within 2° of each other we obtain $p \sim 3 \times 10^{-3}$.

In the 20 years since gamma ray bursters were first discovered there has been only one plausible association, the N49/SGR0526–66 association. In view of this fact, the moderately low value of p , and the fact that G10.0–0.3 is the only interesting radio source in the entire localization box (Fig. 1), further studies of G10.0–0.3 are clearly warranted. High-quality radio images are needed to conclude firmly that the object is an SNR. If SGR1806–20 becomes active again, then it may be possible to improve the localization using the new interplanetary network¹⁶ and confirm its association with G10.0–0.3.

Assuming that SGR1806–20 and G10.0–0.3 are associated, one can draw several conclusions. It is well known that remnants fade rapidly¹⁷ at radio wavelengths on timescales, $\tau_{\text{SNR}} \lesssim 10^4$ yr; for example, the age of N49 is estimated¹⁸ to be 5.4×10^3 yr. Thus the association of SGR0526–66 and SGR1806–20 with remnants strongly suggest that SGRs must be associated with young neutron stars. We assume the number of active SGRs in our Galaxy, $N_{\text{SGR}} \lesssim 10$ and thus derive an SGR birthrate of $B_{\text{SGR}} = N_{\text{SGR}}/\tau_{\text{SNR}} \lesssim 10^{-3} \text{ yr}^{-1}$. This is a few percent of either the non-type-Ia supernovae birthrate¹⁹ or the pulsar birth rate^{19,20}, $B_n \gtrsim 0.02 \text{ yr}^{-1}$. It follows that either (1) all young neutron stars pass through an SGR phase (duration, $N_{\text{SGR}}/B_n \lesssim 5 \times 10^2$ yr), or (2) only a certain fraction ($B_{\text{SGR}}/B_n \lesssim 2\%$) of neutron stars, with special attributes, become SGRs during their youth ($t \lesssim \tau_{\text{SNR}}$), for example, slowly rotating and highly magnetized neutron stars. This model is suggested both by observations of an 8-s periodicity in the anomalous burst from SGR0526–66 and by theoretical arguments^{21,22}.

If model (1) is correct, then pulsations from the young pulsar (typical period, few tens of milliseconds) and the associated plerion should be detectable. The (thermal) X-ray emission from the blast wave in old SNRs like N49 (and presumably G10.0–0.3) is soft, $kT \lesssim 1$ keV, in contrast to the power-law spectrum of the pulsar and the plerion. Detection of hard photons towards N49 and G10.0–0.3 would decisively confirm this model.

The most important conclusion of this paper is that SGRs are probably young neutron stars, still embedded in remnants of their supernovae. If this is accepted, then a number of SGR models (standard pulsars, defunct pulsars) are ruled out. Models attributing the SGR phenomenon to structural changes in (young) pulsars (for example, see ref. 11) are favoured. In this context, it is interesting to consider a recent model²³ in which the GRBs are highly beamed, but distant, versions of SGRs. Depending on their distance, it is possible that future space missions can directly detect the host SNR (assuming that by then GRBs can be accurately localized).

Recently the Compton Gamma Ray Observatory (CGRO) has detected bursts²⁴ from SGR1900+14. No catalogued SNRs are found within the published²⁴ localization function. This does not affect our argument as SNR surveys in this region are quite

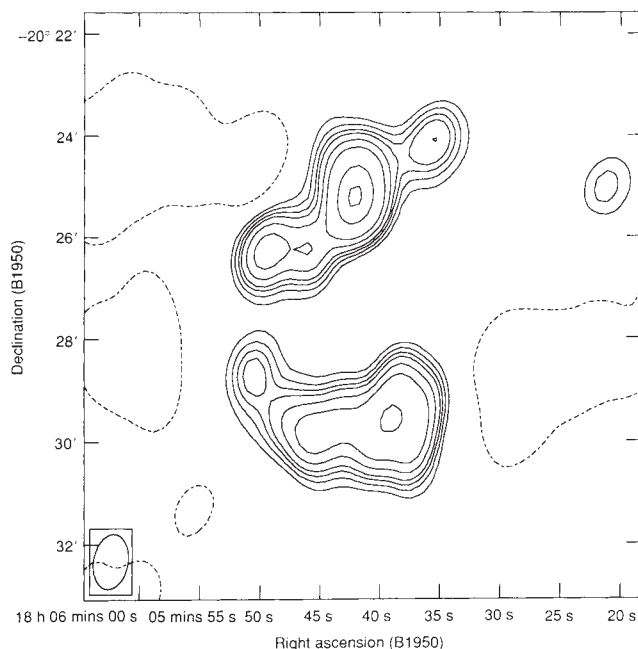


FIG. 2 Radio image of G10.0–0.3 obtained from archival VLA data at 1490 MHz (obtained 21 Jan 1990). Total integration time is 26 min. Contour intervals are plotted at $-2, 2, 3, 4, 5, 7, 10, 20$ and 30 times the r.m.s. noise level of $2.6 \text{ mJy beam}^{-1}$, with negative contours indicated by dashed lines. The resolution of the image ($65'' \times 40''$) is indicated by the size of the beam in the lower left corner. G10.0–0.3 breaks into two components. The faint extensions to the east of each component appear to be real and have counterparts in the original 330 MHz image. These may be part of a larger more diffuse component that is missing in these interferometric images. Unfortunately the image fidelity is limited by a bright H II region 11 arcmin east of G10.0–0.3. The total flux of the object in this image is 0.33 Jy and 1.2 Jy in the 330-MHz image. These values are significantly different from extrapolations of the Molongio–Parkes measurements (see text), consistent with the above discussion that the object is partially resolved even in the low resolution VLA data. Because both the low resolution 1.4 GHz and 330 MHz images have similar resolution, a reasonable estimate of the spectral index, α can be obtained from the VLA total flux measurements. We confirm $\alpha \sim -1$.

incomplete. VLA observations to determine the nature of potential SNR candidates are in progress.

We end with an observation. It is usually assumed, for the purpose of identification of counterparts, that only the total area of the localization matters. But as Equation (1) shows, a localization with a very long axis is undesirable in two situations: (1) extended counterparts (for example, SNRs as discussed above) or (2) when clustering statistics of the bursts are expected to provide crucial clues (for cosmological models, for instance). In the second case, the clustering statistics will be limited to angular scales set by the long axis. Large-area X-ray monitors with masks have the advantage of providing circular localizations with acceptable areas. $\square$

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Entropy-driven formation of a superlattice in a hard-sphere binary mixture

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A MIXTURE of two dissimilar species (A and B) may freeze to form a substitutionally ordered crystal, the structure of which can vary from a lattice with only a few atoms per unit cell to a complex 'superlattice'. For example, a mixture of sodium and zinc can form a solid with the AB_{13} structure with 112 atoms per unit cell¹ (Fig. 1a). One might suspect that very specific energetic interactions are needed to stabilize a structure as complex as this. But recent experiments^{2,3} show that the AB_{13} structure is also formed in mixtures of spherical colloidal particles with different diameters, which interact only via simple repulsive potentials. This raises the possibility that the formation of an AB_{13} superlattice might be supported by entropic effects alone. To investigate this possibility, we present here computer simulations of a binary mixture of hard spheres. Our calculations show that entropy alone is indeed sufficient to stabilize the AB_{13} phase, and that the full phase diagram of this system is surprisingly complex. Our results also suggest that vitrification or slow crystal nucleation in experimental studies of colloidal hard spheres can prevent the formation of equilibrium phases.

To predict the phase diagram of a binary mixture by computer simulation, we must determine the Gibbs free energies of all competing phases as a function of the external pressure (see, for example, ref. 4). On the basis of space-filling arguments⁵, for diameter ratios ($\alpha = \sigma_B/\sigma_A$) in the range 0.5-0.8, the following phases may be expected to occur in a mixture of hard spheres: pure face-centred-cubic (f.c.c.) crystals of either component (A and B), the AB_{13} (Fig. 1a) and AB_2 (Fig. 1b) lattices, and the binary fluid (F). If a given structure has a very high volume fraction at close-packing (ϕ_{cp}), then at lower densities the constituent particles will have a large free volume in which to move and hence a high translational entropy. Murray and Sanders showed⁵ that for $0.5 \leq \alpha \leq 0.8$, ϕ_{cp} is appreciably higher for AB_{13}

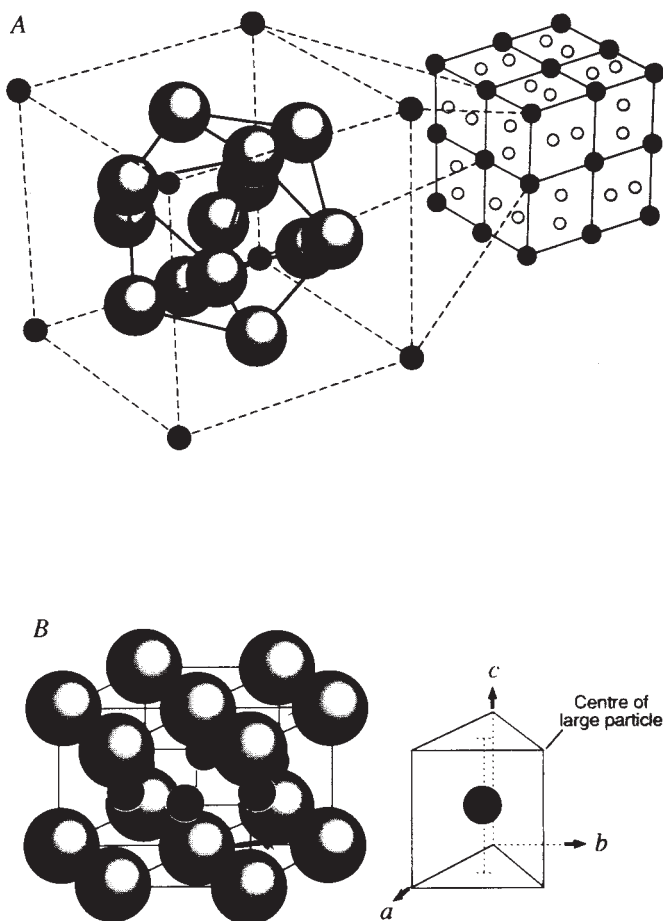


FIG. 1 A, The AB_{13} structure consists of body-centred icosahedral clusters of 13 B particles contained within simple cubic subcells of the larger component (A). The full unit cell (112 atoms) consists of eight subcells with neighbouring icosahedra alternating in orientation by $\pi/2$. B, The AB_2 structure is made up of alternating hexagonal layers of the small and large particles. The large particles (A) form close-packed layers aligning directly above each other along the c axis, while the small particles (B) occupy trigonal prismatic sites between these layers and form planar hexagonal rings resembling the carbon layers in graphite.

and AB_2 than for any other binary crystal structure. The maximum packing fraction of these mixed crystals is, however, comparable to that of the pure A and B phases ($\phi_{cp} = 0.7405$). For α above 0.85 a random alloy with f.c.c. structure is known to be stable^{6,7} and for small values of α (≤ 0.5) a range of interstitial structures (for example NaCl) might be expected to be stable, again on space-filling grounds.

To compute the Gibbs free energy of the possible phases of the mixture, we use a numerical technique called 'thermodynamic integration' (see for example ref. 8). In such calculations, one computes the reversible work needed to transform the phase under consideration into a state of known free energy. To compute the free energy of the binary fluid, we compute the reversible work needed to compress the fluid mixture from zero density (the ideal gas) to the required packing fraction. We have used the semi-empirical expression of Mansoori *et al.*⁹ for the binary fluid equation-of-state, to facilitate such a calculation. Integration from the dilute gas cannot be used for solids, however, because of pronounced hysteresis on encountering the freezing transition. Instead we have used the method of Frenkel and Ladd¹⁰, taking the reference state (of known free energy, F) to be the corresponding Einstein crystal, that is the same crystallographic structure with the particles bound to lattice sites by harmonic springs. These calculations have been described in detail elsewhere^{11,12}.

Figure 2 shows the reduced-pressure/composition (P^*/X) phase diagram for a diameter ratio, $\alpha = 0.58$. At low densities only the binary fluid is stable. However, for compositions $X \leq 0.9$, as the pressure is increased there is a tendency for f.c.c. solid A (large spheres) to be precipitated. At higher pressures still, the crystal structure AB_2 becomes stable in coexistence with either solid A or fluid, and at even greater pressures the AB_{13} superlattice is thermodynamically stable for compositions around its stoichiometric value, $X = 13/14$.

One could still argue that specific energetic contributions (to the effective interatomic interactions) may be responsible for the formation of AB_{13} and AB_2 structures in metallic¹ and molecular¹³⁻¹⁵ mixtures or even in charge-stabilized colloids^{16,17}. For hard-particle systems however, the only non-ideal contributions to the free energy are entropic in origin. Hence, we find that entropy, normally regarded as a driving force for disorder, is responsible for the formation of crystal structures with very complex order.

In the experiments of Bartlett *et al.*^{2,3} the total volume accessible to the colloidal mixture is set by the suspending fluid. Hence,

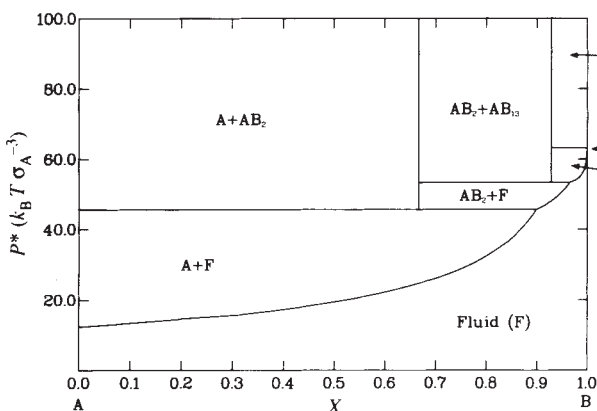


FIG. 2 P - X phase diagram of a binary mixture of hard spheres with a diameter ratio $\alpha = 0.58$. The pressure P^* is given in reduced units $k_B T \sigma_A^{-3}$, where σ_A is the diameter of the large spheres, k_B is the Boltzmann constant, and T is the absolute temperature. In addition to the fluid phase F , we observe the following stable solid phases: pure A, pure B, AB_2 and AB_{13} .

in order to compare our simulations with these experiments, it is more convenient to compute the phase diagram of the mixture at constant total volume. Such a representation of the phase diagram is shown in Fig. 3 where, following ref. 18, we use the partial packing fraction of small spheres (ϕ_B) and large spheres (ϕ_A) as the independent coordinates. A notable feature of this representation is the large amount of (ϕ_B, ϕ_A) -phase space occupied by triangular eutectic (three-phase) regions. In the P - X diagram, these eutectics were only represented by points, in accordance with the Gibbs phase rule. The ratio of the volumes of the phases participating in this three-phase coexistence is equal to the ratio of the areas of the three internal triangles formed between the point considered and the vertices of the three-phase region¹⁸.

In Fig. 3, we have indicated the compositions of the suspensions for which Bartlett *et al.*³ observed the formation of solid phases. The symbols denote the phases they identified from the light-scattering Bragg peaks. In general terms their observations agree well with our thermodynamic phase diagram. The freezing line is well located. In addition, the phase diagram clearly shows why the formation of AB_2 crystals at $X_B = 2/3$ did not occur at the packing fractions reached in the experiments, an observation highlighted by Bartlett *et al.*³

The most striking difference between simulation and experiment is that Bartlett *et al.*³ did not observe formation of solid A at compositions for which it is the thermodynamically favoured phase. At the composition corresponding to the AB_2 stoichiometry, an amorphous solid appeared instead of A. For compositions corresponding to AB_4 and AB_6 , solid AB_2 and fluid (F) was observed, as if the system had avoided the lowest thermodynamic state ($A + F$) and settled into the neighbouring stable phase which has a slightly higher free energy at these compositions. Kinetic factors may be responsible for these discrepancies, that is the inability of the system to nucleate pure A crystallites, perhaps because the composition of the fluid is so different from

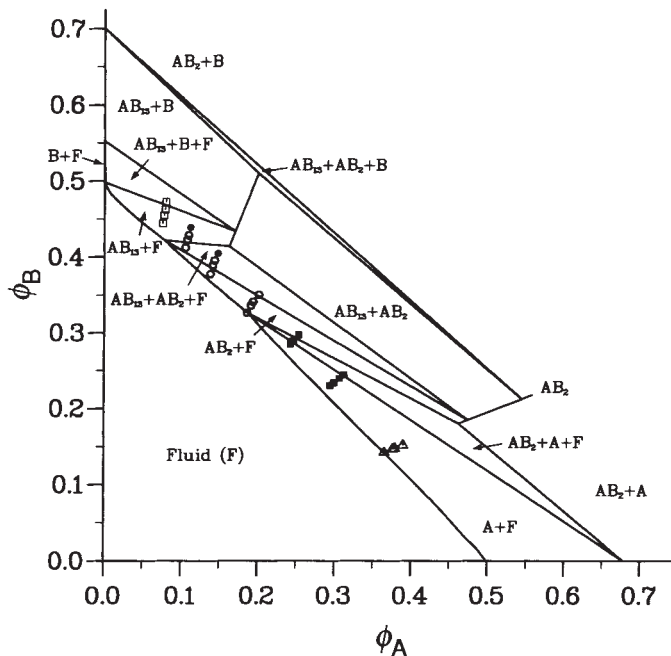


FIG. 3 Phase diagram at constant volume of a binary mixture of hard spheres with a diameter ratio $\alpha = 0.58$. The lines indicate boundaries of two- or three-phase regions. In all cases, the nature of the coexisting phases is indicated. Also shown are the state points studied by Bartlett *et al.*³ for a binary mixture of colloidal particles with the same diameter ratio. The symbols indicate the phases observed in ref. 3: $\square$, $AB_{13} + B + F$; $\circ$, $AB_{13} + F$; $\bullet$, $AB_2 + F$; $\triangle$, amorphous solid.

that of the nucleating solid. (We note that in their earlier paper, Bartlett *et al.*² did observe formation of pure A from A-rich suspensions at $\alpha=0.61$; here crystallization was observed for AB_x compositions where x is less than 1.5 and amorphization was observed for larger x .) A similar reasoning could account for the formation of AB_{13} rather than AB_2 at compositions AB_9 and AB_{14} , especially if the interfacial tension between AB_{13} and the fluid were lower than for AB_2 . This seems likely in view of the high degree of local icosahedral order in dense fluids. AB_{13} has a particularly large number of icosahedral centres, suggesting that the barrier to nucleation may be particularly low. Detailed examination of its structure shows that all the small (B) atoms are icosahedrally coordinated; the central atom of the cluster is surrounded by a perfect icosahedron of B particles and the B particles on the surface by a distorted icosahedron containing two A particles. □

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Stratospheric ozone depletion by $ClONO_2$ photolysis

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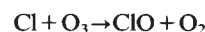
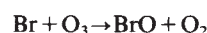
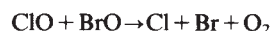
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SPRINGTIME ozone depletion over Antarctica is thought^{1,2} to be due to catalytic cycles involving chlorine monoxide, which is formed as a result of reactions on the surface of polar stratospheric clouds (PSCs). When the PSCs evaporate, ClO in the polar air can react with NO_2 to form the reservoir species $ClONO_2$. High concentrations of $ClONO_2$ can also be found at lower latitudes because of direct transport of polar air or mixing of ClO and NO_2 at the edges of the polar vortex. $ClONO_2$ can take part in an ozone-depleting catalytic cycle¹⁸, but the significance of this cycle has not been clear. Here we present model simulations of ozone concentrations from March to May both within the Arctic vortex and at a mid-latitude Northern Hemisphere site. We find increasing ozone loss from March to May. The $ClONO_2$ cycle seems to be responsible for a significant proportion of the simulated ozone loss. An important aspect of this cycle is that it is not as limited as the other chlorine cycles to the timing and location of PSCs; it may therefore play an important role in ozone depletion at warm middle latitudes.

High levels of $ClONO_2$ have been observed in both hemispheres at the edges of the polar winter vortex^{3,4} and inside the Arctic spring vortex⁵. We show here for two cases that ozone depletion does not cease once $ClONO_2$ is formed: (1) assuming that at high latitudes ($65^\circ N$) all the available inorganic chlorine is in the form of $ClONO_2$ after the PSCs have evaporated in March and (2) assuming that this high-latitude air has mixed with air from low latitudes, performing calculations at a lower latitude ($55^\circ N$). These two cases may be considered representative of air inside and outside the vortex respectively.

The diurnal photochemical box model⁶ we use contains a full description of stratospheric chemistry and has been reformulated so that Cl, ClO, Br and BrO are integrated separately. We therefore make no steady-state assumption for Cl+ClO and Br+BrO. The heterogeneous removal of N_2O_5 by the background aerosol layer is also included. Figure 1 shows ozone depletion at an altitude of 19 km (near the peak of the ozone layer) from March to May, assuming different total chlorine and total bromine concentrations¹ that are representative of the years 1980, 1990 and 2000. After 75 days there is a depletion of ozone of 9.5, 12.5 and 16% for these respective years. This is a significant additional change if we consider that models⁷ calculate about 20% chemical depletion of ozone from December to March for a 1990 atmosphere. We therefore conclude that there is significant additional ozone depletion months after heterogeneous reactions have ceased. Comparing the 1990 run with the 1980 run shows that ozone is 3.3% lower in 1990, which is about half of the observed column ozone trend for these years¹. The calculations do not account for mixing and the calculated ozone loss is therefore an upper limit.

The two main ozone depletion cycles are now considered.



The rate of this catalytic cycle⁸ is determined by the reaction $ClO + BrO$. Ozone can also be destroyed by $ClONO_2$ in the following catalytic cycle.

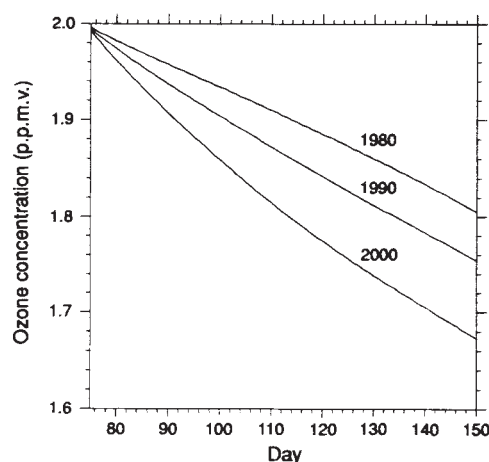
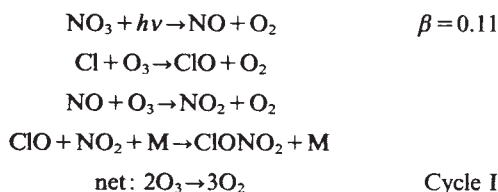


FIG. 1 The concentration of ozone at an altitude of 19 km, $65^\circ N$, as a function of time (day 75 = 16 March). Initial (day 74) conditions (in volume mixing ratio) for the years 1980, 1990 and 2000: $ClONO_2 = 2, 3, 4 \times 10^{-9}$; $BrO = 10, 15, 23 \times 10^{-12}$; $HNO_3 = 10, 9, 8 \times 10^{-9}$; for all years: $CH_4 = 0.5 \times 10^{-6}$, $H_2O = 5 \times 10^{-6}$, $H_2 = 0.5 \times 10^{-6}$, $CO = 2 \times 10^{-8}$, $H_2O_2 = 1 \times 10^{-12}$, $ClO = HCl = NO = NO_2 = N_2O_5 = BrONO_2 = CH_3O_2 = 0$, $T = 210 K$, $p = 64.5 mb$. We have specified a rate constant for $N_2O_5(g) + H_2O(l) \rightarrow 2HNO_3(g)$ of $4 \times 10^{-6} s^{-1}$ on the sulphate aerosol layer¹.



where $\text{M} = \text{N}_2 + \text{O}_2$, α is the yield of $\text{Cl} + \text{NO}_3$ from ClONO_2 photolysis⁹ and may be less than 0.9 at atmospherically opaque wavelengths¹⁰ and β is the yield of $\text{NO} + \text{O}_2$ from NO_3 photolysis¹¹. The other photolysis channels, which yield $\text{O} + \text{ClONO}$ and $\text{NO}_2 + \text{O}$ respectively, form null cycles. The rate of cycle I can be calculated as $2\alpha\beta J[\text{ClONO}_2]$, where J is the photodissociation coefficient of ClONO_2 . At noon (solar zenith angle = 48° , day 130 (May 10)) the lifetime ($1/J$) of ClONO_2 at 19 km is about 5 hours.

Figure 2 shows the change of mixing ratio of some key species for the 1990 run. During spring, ClO and ClONO_2 are converted to HCl . However, ClO decreases much more rapidly than the concentrations of ClONO_2 , which is >1 part per billion by volume (p.p.b.v.) even 75 days after the initialization. This explains the dominance of cycle I (see below) over all other ozone destruction cycles involving chlorine, once ClO is less than 100 parts per trillion by volume (p.p.t.v.) and the ratio of $\text{ClONO}_2/\text{ClO}$ is greater than about 20. The rate of increase in HCl is initially fast, but is much slower towards the end of the run. The rate of conversion of ClO and ClONO_2 to HCl depends largely on the sequence of reactions $\text{ClO} + \text{NO} \rightarrow \text{Cl} + \text{NO}_2$ and $\text{Cl} + \text{CH}_4 \rightarrow \text{HCl} + \text{CH}_3$. As HNO_3 is photolysed, NO and NO_2 increase during the runs, leading to HCl formation. The rate of production of ClONO_2 depends on the product of ClO and NO_2 and decreases only slowly during the run. This explains the long time it takes to convert ClONO_2 to (inactive) HCl . Ozone destruction continues until this conversion is complete.

To determine the cause of ozone depletion, we have calculated the rates of chlorine catalytic cycles. Figure 3a shows that most of the early ozone decrease is due to the $\text{ClO} + \text{BrO}$ cycle⁸. As ClO is less than 200 p.p.t.v. for these runs, the dimer (Cl_2O_2) ozone-loss cycle¹² is of only minor importance¹. By April, cycle I is the dominant chlorine cycle. Because of the long time it takes to convert ClONO_2 to HCl , this cycle outlasts all other chlorine cycles. When comparing the loss rates due to the various cycles during the day we find that the relative importance of cycle I is smallest at noon. The other cycles show a larger diurnal variation.

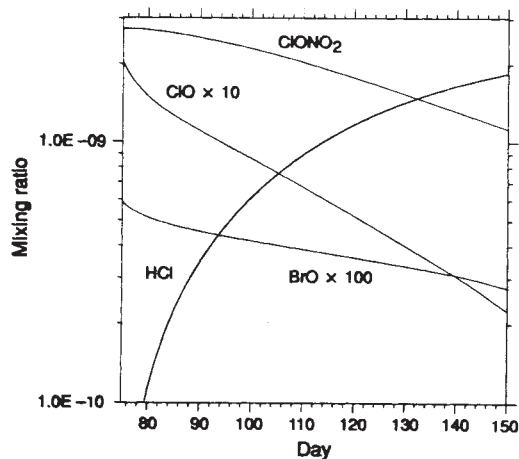


FIG. 2 Noon mixing ratio of ClO , HCl , ClONO_2 and BrO as a function of time (in days) for the 1990 run, at an altitude of 19 km, 65°N . By day 150, $\text{NO} + \text{NO}_2 = 2.4 \times 10^{-9}$.

There are some limitations in using a box model over long timescales as we do here. We are not suggesting that air is unmixed or remains at one particular altitude and latitude for 75 days. Using the same 1990 initialization conditions at a higher altitude (22 km), cycle I is initially the third most important cycle, after the $\text{ClO} + \text{O}$ and $\text{ClO} + \text{BrO}$ cycles. By day 120 cycle I again becomes the dominant chlorine cycle, but oxygen photolysis is also much faster so that ozone begins to increase after a maximum depletion of 9%. At 16 km, cycle I dominates all other chlorine cycles after day 90 and ozone is reduced by 10%. We can therefore conclude that cycle I is important throughout the lower stratosphere, but that its importance decreases with increasing altitude.

In spring, there is horizontal and vertical mixing on timescales of less than 75 days, which the model does not account for. This may be why simulated NO_x and NO_y concentrations at the end of the model run appear rather larger than low-latitude observations¹³ would suggest. To see how this might affect ozone

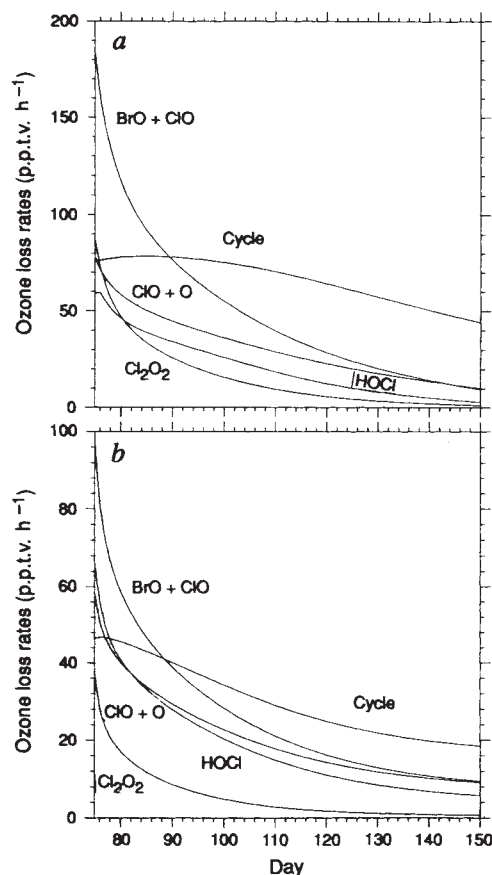


FIG. 3 Hourly ozone loss rate at noon for the 1990 run at 19 km altitude, due to various chlorine-catalysed cycles as follows. ' Cl_2O_2 ' is the dimer¹² loss rate calculated as $2J[\text{Cl}_2\text{O}_2]$: $\text{ClO} + \text{ClO} + \text{M} \rightarrow \text{Cl}_2\text{O}_2 + \text{M}$, $\text{Cl}_2\text{O}_2 + h\nu \rightarrow 2\text{Cl} + \text{O}_2$, $2 \times (\text{Cl} + \text{O}_3 \rightarrow \text{ClO} + \text{O}_2)$. ' $\text{ClO} + \text{O}$ ', loss calculated as $2k[\text{ClO}][\text{O}]$ (ref. 15): $\text{Cl} + \text{O}_3 \rightarrow \text{ClO} + \text{O}_2$, $\text{ClO} + \text{O} \rightarrow \text{Cl} + \text{O}_2$. ' $\text{ClO} + \text{BrO}$ ', loss calculated as $2k[\text{ClO}][\text{BrO}]$ (ref. 8) and includes the BrCl photolysis channel. ' HOCl ', loss calculated as $2J[\text{HOCl}]$ (ref. 16): $\text{HOCl} + h\nu \rightarrow \text{OH} + \text{Cl}$, $\text{Cl} + \text{O}_3 \rightarrow \text{ClO} + \text{O}_2$, $\text{OH} + \text{O}_3 \rightarrow \text{HO}_2 + \text{O}_2$, $\text{HO}_2 + \text{ClO} \rightarrow \text{HOCl} + \text{O}_2$. 'Cycle I' calculated as $2\alpha\beta J[\text{ClONO}_2]$ (see text). k and J are the respective rate constants and photodissociation coefficients¹⁷. a, Values for 65°N . b, Values for a 'mixed' initialization at 55°N : $\text{ClONO}_2 = 1.6 \times 10^{-9}$, $\text{HCl} = 0.4 \times 10^{-9}$, $\text{BrO} = 12 \times 10^{-12}$, $\text{HNO}_3 = 6.3 \times 10^{-9}$, $\text{CH}_4 = 1 \times 10^{-6}$, $\text{H}_2\text{O} = 4.5 \times 10^{-6}$ all other species as in 1990 run (Fig. 1). By day 150: $\text{O}_3 = 1.86 \times 10^{-6}$, $\text{ClONO}_2 = 0.4 \times 10^{-9}$, $\text{HCl} = 1.6 \times 10^{-9}$, $\text{NO} + \text{NO}_2 = 1.3 \times 10^{-9}$.

depletion at mid-latitudes, we performed a run at 55° N, using an average of high- and low-altitude air¹⁴ to represent the mixing of the two air masses. The 'mixed' initialization is lower in ClONO₂ and higher in HCl. Figure 3b, shows the ozone loss rates for the various chlorine cycles at 55° N. The absolute loss rates are smaller, yet ozone is still reduced by 7% over the 3 months considered. As in the high latitude case, initial destruction is dominated by the BrO + ClO cycle, but cycle I eventually dominates. This calculation shows that the relative importance of cycle I is not diminished by mixing of air. When we compare mixed air there is a difference of only 1.6% between 1990 and 1980, compared with the 3.3% obtained previously. Mixing of air therefore reduces local loss rates and the trend in ozone. However, mixing by averaging of these two air masses constitutes a doubling of the volume, so that the ozone trend for the larger volume is similar for the mixed and unmixed air masses.

Cycle I will also play a role in explaining the winter ozone loss over the past decade¹. All heterogeneous ClO activation will eventually form ClONO₂. During the polar winter, high levels of ClONO₂ are seen at the edge of the polar vortex in both hemispheres^{3,4}. From 1 January to 15 March the model, with a high-latitude initialization (as above), calculates an ozone reduction of 8.5% at 60° N (the vortex may extend further south during the winter). Most of this depletion is by the ClO + BrO cycle, but by early February cycle I is the second most important cycle. This 'collar' region could also mix with mid-latitude air and cause ozone depletion outside the vortex. For a run (1 January to 15 March) with a 'mixed' initialization (as above) at 50° N, most of the ozone depletion of 7% is by the ClO + BrO cycle, but by mid-February cycle I is the second most important chlorine cycle. Although we focus our attention on the previously unrecognized importance of direct ozone destruction by ClONO₂ photolysis, we note that the indirect effect of releasing ClO by ClONO₂ photolysis is also very important for ozone loss.

The calculations done here are also representative of the Southern Hemisphere. However, rapid ozone depletion can only be explained² by high levels of ClO. Cycle I is too slow, especially as any ClONO₂ would rapidly react with cloud particles. The conversion of ClO to ClONO₂ may be limited by denitrification (which is less common in the Arctic) in the vortex core. Nevertheless, high levels of ClONO₂ can be expected at the edges of the vortex.

Despite the limitation of using a box model here, a qualitative picture of ozone destruction emerges. Every winter heterogeneous reactions in the polar regions of both hemispheres convert the chlorine reservoirs to ClO. There are three stages for ozone depletion. First, the dimer mechanism causes most of the early depletion in polar regions when ClO concentrations are high. Second, when ClO concentrations fall, the ClO + BrO cycle dominates ozone loss. Third, as the concentration of ClONO₂ increases, cycle I causes further depletion. Air that is high in ClONO₂ can be transported to mid-latitudes and cause ozone destruction. The conversion of ClONO₂ to inactive HCl is slow, so that prolonged periods of ozone depletion by cycle I can occur. Unlike the other mechanisms, cycle I is therefore not restricted to the timing and location of PSCs. It is also noteworthy that ozone depletion later in the season will be more damaging to the biosphere as the ultraviolet-light flux is largest in the summer. □

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Theoretical determination that electrons act as anions in the electride Cs⁺(15-crown-5)₂·e⁻

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ELECTRIDES are crystalline salts formed from complexed alkali-metal cations. There has been some dispute as to whether the valence electron from the alkali ion becomes a trapped interstitial anion^{1,2} or resides at or near the alkali-metal nucleus³. If the former description holds, electrides would represent stoichiometric counterparts of ionic insulators containing 'F-centre' electronic defects. Experiments^{1,2} have so far failed to resolve the question. Here we present *ab initio* self-consistent density-functional calculations⁴ of the electron distribution in the electride Cs⁺(15-crown-5)₂·e⁻. We find that a spatially localized electron is located at the anion site, in accord with the F-centre model. Although the potential is in fact repulsive in this region, the electron is apparently forced to reside here by the need to lower its kinetic energy. We suggest that this picture may hold for other electrides as well.

The novel properties of the electrides and alkaliides depend on their unusual structural features. The electride Cs⁺(15-crown-5)₂·e⁻ considered here crystallizes in a triclinic *P1* structure⁵ with *a* = 8.597, *b* = 8.886 and *c* = 9.941 Å, *α* = 102.91°, *β* = 90.06°, and *γ* = 97.74°. The unit cell, which may be viewed as distorted simple cube, contains one formula unit (71 atoms) with the Cs cation at the (0, 0, 0) position sandwiched between two crown ether molecules, 5(CH₂CH₂O)≡(15-crown-5). There is a large cavity centred at (1/2, 1/2, 1/2), which is the presumed site of the trapped electron (and of the negative alkali-metal atom in the corresponding alkaliide Cs⁺(15-crown-5)₂·Na⁻). The enveloping complexant structure presumably stabilizes the solid by isolating the cation and preventing its recombination with the trapped electron. The measured magnetic susceptibility exhibits Curie–Weiss behaviour from ~5–260 K, where there is a structural phase transition, and upon cooling below ~4.6 K, where there is an antiferromagnetic transition⁶. This is the only known electride exhibiting an antiferromagnetic transition, and its low transition temperature is consistent with small coupling between trapped electrons.

Self-consistent local density functional approximation (LDA) calculations were carried out using a new *ab initio* mixed-basis method⁴ that employed a basis set consisting of 12,219 plane waves and 187 local basis functions, and 100 occupied bands, including the Cs 5s- and 5p-derived states, which were found to be hybridized and band-like. (The local basis functions are constructed as described in ref. 4. There are three radial functions (each) for Cs *s*, *p* and *d* character, one (each) for *s* and *p* character on the C and O atoms and a single *s* function on each H atom. The Cs 4*d*, 5*s* and 5*p* states were retained as

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variational states along with the usual valence states.) As the calculated electronic energy bands are very complicated, they are schematically represented in Fig. 1, which shows an occupied manifold of bonding molecular orbitals that is separated from an unoccupied manifold of anti-bonding molecular orbitals by an energy 'gap' of ~ 5 eV. In addition, a narrow electrider energy band (width ~ 0.15 eV), originating from the Cs 6s electron and containing exactly one electron, lies in this 'gap' about 0.4 eV below the unoccupied manifold. The dispersion of this state is highly anisotropic (Table 1). The largest band dispersion from the zone centre occurs along the *b* axis. Hybridization with the unoccupied molecular states complicates the dispersion of this state, however, so that simple single orbital tight binding fits do not model it.

Figure 2 shows the charge density of this electrider level as a sequence of constant-density surfaces, corresponding to densities that range from 2.0 to 7.0×10^{-4} e per a.u.³, the average density of a single electron in the unit cell being 2.02×10^{-4} e per a.u.³ (where an atomic unit, a.u., is equivalent to the Bohr radius, or 0.529 Å). This density was obtained from the wavefunction at the centre of the Brillouin zone. Minor differences are seen at other points in the zone. The volume shown is a cube of edge 24.0 e.a.u., ~ 2.8 times the volume of a single unit cell. The charge density of the electrider state is centred in the cavity at $(1/2, 1/2, 1/2)$ and reaches a maximum (9.5×10^{-4} e per a.u.³) there. At the highest density shown in Fig. 2, the electrider state forms isolated pockets located in the central cavities. The 5.0×10^{-4} e per a.u.³ surface reveals a narrow one-dimensional channel that has opened up along the *b* axis, connecting the cavity to those in neighbouring cells. A narrower channel is also present along the *a* directions, but none opens up along the *c* axis. As seen in Table 1, the *k*-space dispersion in the $\langle 1, 0, 0 \rangle$ and $\langle 0, 0, 1 \rangle$ directions is only $\sim 20\%$ of that along $\langle 0, 1, 0 \rangle$, reflecting the greater overlap of the electrider state charge density in the *b*-axis channel.

The behaviour of the self-consistent potential (Fig. 3) sheds light on the formation and properties of the electrider state. Surprisingly, the potential at the centre of the cavity is a maximum and not a relative minimum, as has been suggested,⁷ nor is there a potential 'barrier' set up by the crown ether molecules enveloping the alkali-metal cation⁸. The potential instead is

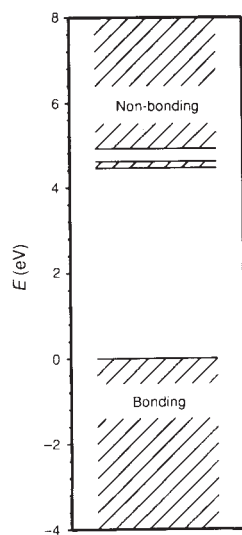


FIG. 1 Schematic representation of the electronic energy bands. The energy zero is the valence band maximum (E_v) the half-filled electrider state is depicted by the narrow band at ~ 4.5 eV, and the conduction band minimum (E_c) is at ~ 4.9 eV.

TABLE 1 Brillouin zone *k*-dispersions

<i>k</i> -point	E_v	E_{elec} (eV)	E_c (eV)
0, 0, 0	-0.01	4.48	5.32
1/2, 0, 0	0.00	4.47	5.20
0, 1/2, 0	-0.03	4.55	4.98
0, 0, 1/2	-0.03	4.46	5.13
1/2, 1/2, 1/2	-0.02	4.61	4.92

Values calculated using the procedure described in the text. E_v is the valence band maximum, E_{elec} is the electrider state and E_c is the conduction band minimum. The *k*-points are given in reciprocal lattice units, the three directions corresponding to the *a*, *b* and *c* axes respectively.

attractive only near the atomic sites. A cavity with an attractive potential at its centre might also have been surmised from Fig. 2. Instead, the potential is repulsive everywhere (that is, a maximum) in the cavity, forming a plateau along the *b*-axis channel, and decreasing monotonically towards the complexant molecules. Why is the electrider state located in a region where the potential is repulsive? One reason is the required orthogonality of this state to the large manifold of bonding molecular orbitals. This forces the electrider state to reduce its kinetic energy by avoiding the regions in the vicinity of the atoms where the potential is most attractive. Nevertheless, one might still expect it to be centred on the Cs cation, as a sort of 'halo' around the complexant. Because of the presence of the large cavity in this structure, however, the electron (whose energy is 0.7 eV above the plateau in the potential) can lower its kinetic energy most effectively by spreading out into this relatively spacious region where the potential is flat. Ultimately, however, the electron remains bound within the solid by the long-range Coulomb tails of the potential.

In spite of the lack of any formal guarantee, the local density bands can ordinarily be used to predict metallic or insulating behaviour. But when bands as flat as the electrider band (0.15 eV wide) are involved, it is commonly found that a more sophisticated theory of the excitations is required. Optical absorption and d.c. conductivity measurements indicate that this electrider is insulating, with a band gap⁵ of ~ 1 eV. Electron-electron interactions are often important in narrow bands, and may be responsible for the observed insulating behaviour. A good analogy is the partially filled narrow *f* bands in rare-earth compounds that are nevertheless insulating. The Hubbard model, with an atomic-like state at each lattice site, is usually invoked to describe correlation effects in such systems.

In the Hubbard model this state acquires a band width, $W = 2Zt$, where *t* is a matrix element due to interactions with nearest-neighbour states, and *Z* is the number of nearest neighbours contributing to the band width. An additional energy cost, *U*, is imposed for occupying the atomic state with two electrons (which must have opposite spins). For one electron per lattice site, a half-filled metallic band is recovered if $U=0$. For large U/W , one obtains an insulating state with a lower fully occupied band and a higher-lying unoccupied band split by the energy *U*. In the large U/W limit, the low-energy excitations in the Hubbard model are given by an equivalent Heisenberg model with exchange integral $J = 4t^2/U$ (ref. 9). Measured magnetic susceptibility measurements near the antiferromagnetic phase transition have extracted values of the exchange integral in the range $J = 1.6$ – 1.9 K (ref. 5), and this can be used to estimate *U*. In view, however, of the strongly anisotropic character of the calculated band structure, anisotropic exchange couplings are also expected. Assuming that the antiferromagnetic transition is dominated by the smallest *J* value, the smallest calculated band width (that is, along the *a*-axis) is used to extract $t_{small} = W_{small}/4$ and $U \sim 0.15$ eV, using $J = 1.8$ K. The Mott-Hubbard transition

FIG. 2 The charge density of the electride state represented by four constant-density surfaces. The densities are 2.0 (upper left), 3.5 (upper right), 5.0 (lower left) and 7.0 (lower right) $\times 10^{-4}$ e per a.u.³. The atoms are represented by coloured spheres: caesium, green; carbon, red; oxygen, blue; and hydrogen, yellow. The *a* axis is perpendicular to the figure, the *b* axis is vertical, and the *c* axis horizontal.

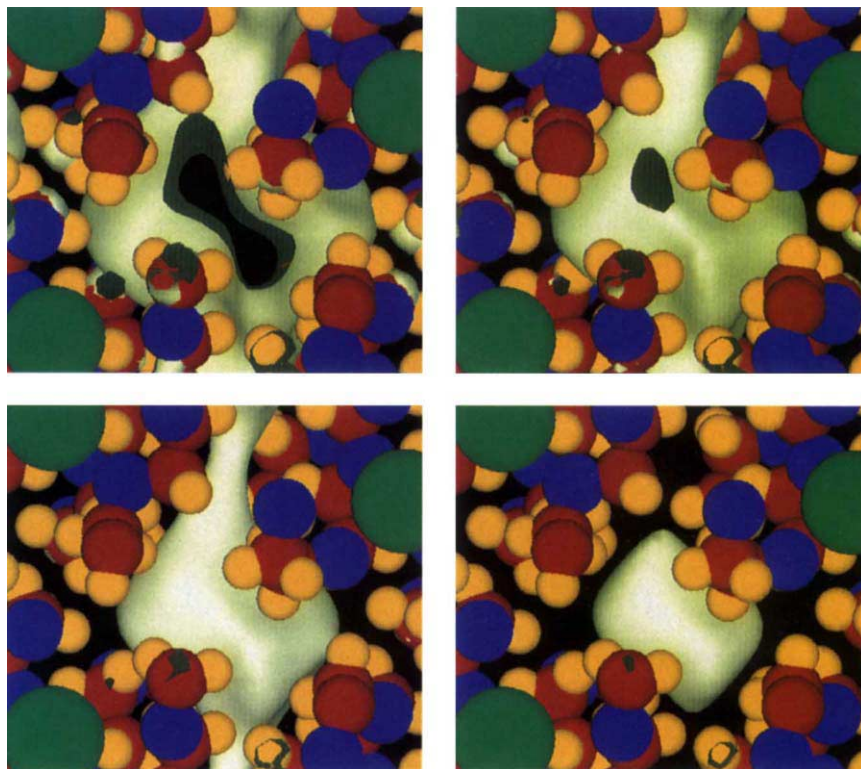
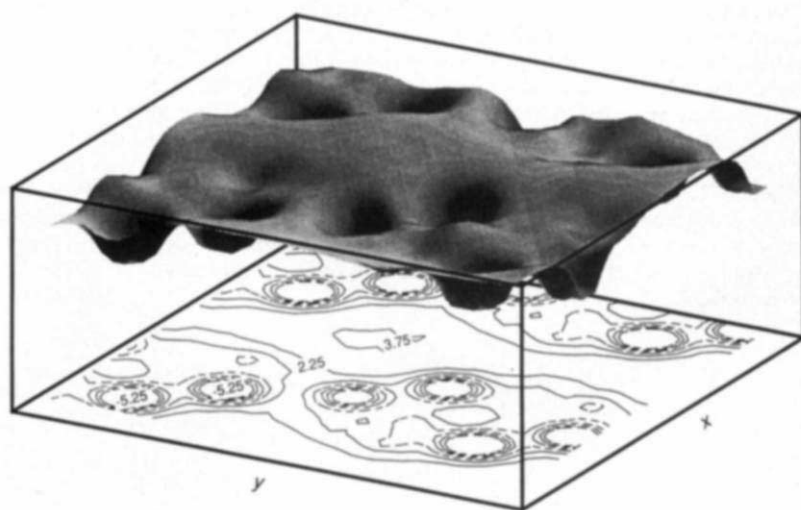


FIG. 3 The self-consistent LDA potential is shown as a perspective surface and contour plot for a plane that cuts through the centre of the cavity and is approximately perpendicular to the *c* axis. The *x* direction in the figure coincides with the *a* axis, and the *y* direction is nearly parallel to the *b* axis. The contour levels run from -5.25 eV to 3.75 eV in uniform steps of 1.5 eV.



is governed by the ratio $U/W_{\text{large}} \sim 1$, where we have used $W_{\text{large}} = 0.15$ eV, which is the maximum band width. Given the large anisotropy of the band structure, and the half filling of the electride band, this size of U/W_{large} may well result in an insulating state within the Hubbard model.

Within such a scheme the electride state is split into an occupied lower Hubbard band and an unoccupied upper Hubbard band. Both the optical gap and our estimate of U are much smaller than the gap (~ 4.5 eV) between the occupied molecular states and the electride band (Fig. 1). Therefore, the occupied lower Hubbard band, like the original electride band, must be located in the gap between the occupied and unoccupied manifolds, and thus retains its interstitial character as calculated within the LDA. Moreover, even details of the LDA density are expected to be preserved. One might compare the copper oxide superconductors, such as La_2CuO_4 : these materials are insulators but have a metallic band structure within the LDA¹⁰. None-

theless, the charge density and even the density response are quite well modelled by the LDA, as evidenced by calculated structural parameters and phonon frequencies.¹¹

The novel structural features of $\text{Cs}^+(15\text{-crown-5})_2 \cdot \text{e}^-$, that is complexed alkali-metal cations and large cavities, are shared by the other known electrides. On the basis of the structural similarities and the behaviour we calculate, we suggest that the existence of an interstitial electron may be a general feature of these materials. Because of the relatively simple electronic structure near the Fermi energy (a single band lying in a large gap), electrides may represent good materials for the study of metal-insulator transitions in general. $\square$

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Solid carbon dioxide in a natural diamond

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CARBON and hydrogen are only trace constituents of the Earth's mantle, yet carbon- and hydrogen-bearing fluids have an important effect on magma genesis, mantle rheology and mantle chemistry. Many mantle-derived rocks record interactions with such fluids, but direct samples of the fluids themselves are rare. Diamonds, owing to their robust nature, constitute effective sampling devices for such deep-seated fluids¹; for example, carbonates and water have been found^{2,3} in fluid inclusions in fibrous diamonds⁴. In addition, CO₂, H₂O, CO, CH₄, H₂ and N₂ have been detected in gases released from diamonds by crushing⁵ or heating^{6,7}, but their primary nature could not be confirmed beyond doubt¹. Here we report the discovery of solid CO₂ in a natural diamond. Infrared spectroscopy indicates that the CO₂ is presently at a pressure of 5 GPa (50 kbar), and must therefore have been trapped at even greater pressures in the hot mantle, corresponding to depths of about 220-270 km. At these pressures, free CO₂ should react with olivine and pyroxene; thus, its survival indicates the presence at depth of an environment of different mineralogy such as a fully carbonated metasomatic vein, or a block of subducted sediments.

FIG. 1 Diamond containing solid CO₂. The brownish-yellow coloured diamond weighs 9.2 mg and was purchased in Israel; the location of its source could not be confirmed. Concentrations of CO₂ are highest in the intensely coloured zone (left side) and are lowest in the weakly coloured zones. The facets of both crystals are octahedral and are populated by many trigonal etch pits (trigons). A few small (2-10 µm) colourless mineral inclusions reside in the intergrown crystals (scale bar = 1 mm).



Carbon dioxide is the most abundant volatile component in fluid inclusions in mantle xenoliths, but because of the limited strength of the silicate minerals, the pressure of trapping rarely exceeds 1 GPa (ref. 1). At greater depths, CO₂ reacts with peridotites to form carbonates, but except for the carbonates associated with kimberlites¹ (that equilibrated at low pressures), only rare samples of mantle carbonates are known^{8,9}. Xenoliths of carbonated peridotites were never documented, probably because they decompose during ascent¹⁰. Recently, small quantities of CO₂ were found together with water, carbonates and silicates in polyphase micro-inclusions in fibrous diamonds^{3,4}.

We report here the discovery of CO₂ in the infrared spectrum of a brownish-yellow diamond (Fig. 1). The diamond consists of two intergrown crystals. The larger one is zoned: its centre is only slightly coloured, while the colour of the outer zone is much more intense. The smaller intergrown crystal is colourless. Infrared absorption spectra of the different zones of the diamond show the common type IaA bands¹¹ with low contents of nitrogen and hydrogen (bands at 1,282 and 3,107 cm⁻¹, respectively^{11,12}; see Fig. 2). Spectra of the coloured zones show four additional bands at 650, 2,376, 3,620 and 3,752 cm⁻¹. The bands at 2,376 and 650 cm⁻¹ dominate the spectrum and their intensity exceeds even that of the diamond absorption bands. These lines are well known as the ν_3 and ν_2 bands of CO₂. The 3,620 and 3,752 cm⁻¹ bands correspond to the combination bands ($\nu_3 + 2\nu_2$ and $\nu_3 + \nu_1$) of CO₂. Close examination reveals that the lines are shifted from the positions at which they occur at one atmosphere pressure. Comparison with high-pressure spectral data of solid CO₂ (ref. 13) reveals that the positions of three of these peaks in the spectrum of the diamond closely fit the spectrum of solid CO₂ at a pressure of 5 ± 0.5 GPa (Fig. 2). The peak at 650 cm⁻¹ also shows a shift towards high pressure, but owing to its strong asymmetry and its lower pressure dependence it yields a lower and much less precise pressure estimation.

Optical examination of the coloured zones under high magnification revealed no visible inclusions. However, the similarity to the spectrum of solid CO₂ implies the presence of a crystalline structure, and hence a distinct assemblage of CO₂ molecules. We therefore assume that the CO₂ resides in submicroscopic inclusions. Future transmission electron microscopy may answer this question. Infrared spectroscopy detected no other impurity in the diamond. Water, CH₄, CO, carbonates and silicates are all infrared-active and the absence of any of their spectral lines

shows that they cannot be present in any appreciable amount. Nitrogen and H_2 are infrared-inactive and their presence cannot be ruled out. However, the concentrations of hydrogen and nitrogen in the diamond matrix decrease with the increasing intensity of the CO_2 bands. Absorption spectra taken in the visible range at room temperature show only a continuous increase in the absorption towards the ultraviolet (typical of type IaA diamonds¹⁴). We found a general positive correlation between the intensity of the brownish-yellow colour and the height of the infrared bands of the CO_2 . Carbon dioxide itself does not absorb in the visible or the near ultraviolet, and the brownish-yellow colour is probably due to the effect of the inclusions on the diamond matrix.

If the CO_2 resides in submicroscopic inclusions, then the internal pressure of 5 GPa is the highest pressure ever measured in any inclusion at room temperature. The pressure at the source region of these diamonds must have been even higher. It can be estimated using the equation of state of CO_2 . We used the equation of state of solid CO_2 (ref. 15) to determine a molar volume of $21 \pm 0.4 \text{ cm}^3 \text{ mol}^{-1}$ at room temperature and $5.0 \pm 0.5 \text{ GPa}$. Following the procedure outlined elsewhere¹⁶, we calculated an increase of less than 1.2% in the volume of the inclusions during the transition from the pressure and temperature of the mantle to the surface. This change is smaller than the uncertainty associated with the molar volume, so we calculated the pressure at high temperatures assuming a constant molar volume. Most of the currently available equations of state for fluid CO_2 include a fitted volume term (adjusted to fit low-pressure experimental data) and cannot be extrapolated to the small molar volume needed here. We found only two equations that were in a parametric form^{17,18} and could be extrapolated to high pressures. Moreover, very high-pressure data from shock-wave experiments were used in adjusting the parameters of these two equations. Both equations of state yield similar curves (Fig. 3) and intersect the shield geotherm¹⁹ at 7–8.5 GPa (70–85 kbar). This pressure range represents either the depth of origin of the diamond, or the level of its last recrystallization event. It is higher than most reported pressure estimates for diamond formation²⁰.

In spite of the uncertainties in the calculated pressure of origin, it is clear that CO_2 was present, as a free stable phase, at pressures in excess of 5 GPa. This is a very surprising result, as CO_2 should react with common upper-mantle rocks (for example, peridotites, eclogites or pyroxenites) at these pressures. As can be seen in Fig. 3, CO_2 should react with olivine in a peridotitic mantle at relatively low pressures according to the following reactions^{21–23}.

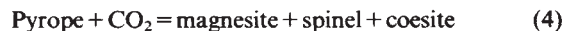


Experiments in more complex systems^{24,25} suggest that melting of natural CO_2 -bearing peridotite occurs at even lower temperatures, so that the stability field of CO_2 in peridotite is even more restricted than in the presence of olivine alone. In any case, CO_2 must react with peridotite to form carbonates or melts at pressures that are much lower than those of the diamond stability field.

In eclogites or pyroxenites, which are olivine-free, CO_2 is stable to higher pressures, but finally reacts with pyroxenes as follows^{23,26}



The curves of the two reactions are almost identical and are represented by a single curve in Fig. 3. The stability field of pyroxene + CO_2 does penetrate the low-pressure region of the diamond stability field, but does not reach the calculated P - T range of the trapped CO_2 . Garnet may be the only common mantle phase that can coexist with CO_2 at the high pressures implied by the isochore of the trapped CO_2 . Extrapolation of low-pressure data for the reaction²⁷



suggests that garnet + CO_2 may be stable to pressures that are ~1–2 GPa greater than the stability limit of pyroxene + CO_2 .

In a peridotitic or eclogitic mantle all olivine and pyroxene should be carbonated before free CO_2 can be stable. Such carbonation involves the addition of >10% of CO_2 (by weight) to eclogite and more than 30 wt% to peridotite. These amounts are orders of magnitudes higher than the normal carbon content of the mantle, but carbonation may be achieved locally, for example, inside a vein of a CO_2 -bearing melt. Carbon dioxide is highly soluble in kimberlitic melts. Its solubility increases substantially with pressure and may reach more than 30 wt% at 6 GPa²⁸. Thus, kimberlitic melts may concentrate carbon from large regions in the mantle and release it locally as they ascend. The released CO_2 reacts immediately with the surrounding mantle, but after a layer of carbonated peridotite (or eclogite) has formed, CO_2 may exist as a free phase. The carbon for the formation of the diamond may come from local reduced carbon or from partial reduction of the CO_2 itself.

Alternatively, the diamond may have formed in an olivine- and pyroxene-free environment, for example, a block of sub-

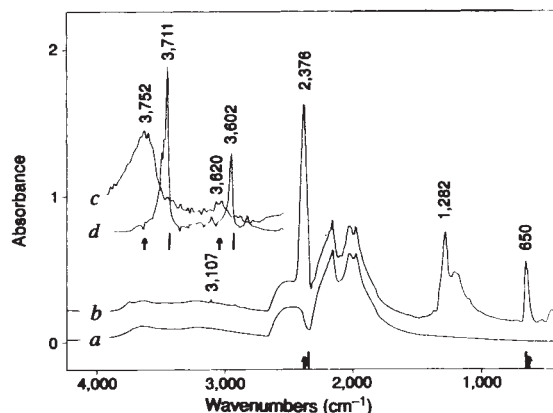
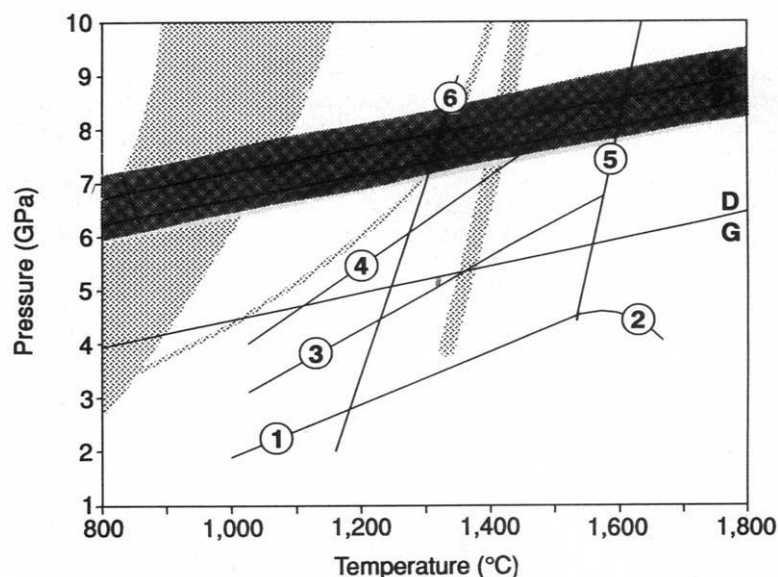


FIG. 2 Infrared absorption spectra. In addition to the intrinsic diamond modes (a, pure type II, nitrogen-free diamond), the spectrum of the most intensely coloured zone of the CO_2 -bearing diamond (b) shows bands due to nitrogen ($1,282$ and $1,220 \text{ cm}^{-1}$; ref. 11), hydrogen ($3,107 \text{ cm}^{-1}$; ref. 12) and CO_2 ($3,752$, $3,620$, $2,376$ and 650 cm^{-1}). Whereas the nitrogen and hydrogen bands appear at their normal (room temperature and pressure) positions, all the CO_2 lines are shifted. Spectrum (c) presents the details of the net (b-a) absorption due to the high energy bands of the CO_2 . The bands are far from the 1 atm position of solid CO_2 (|) or even the 6 kbar positions (spectrum (d); ref. 13) and fall close to the calculated positions for 5 GPa (†). The peak positions of the individual bands correspond to pressures of 5.5 ± 0.3 , 4.7 ± 0.6 , 5.0 ± 0.4 and $3.5 \pm 1.5 \text{ GPa}$ for the $3,752$, $3,620$, $2,376$ and 650 cm^{-1} bands, respectively. The large deviation of the 650 cm^{-1} band is probably the result of its low pressure-dependence and strong asymmetric shape, and is not included in the calculated mean result of $5 \pm 0.5 \text{ GPa}$. Spectra were recorded with a resolution of 4 cm^{-1} using a Nicolet 740 FTIR spectrometer at the Institute of Earth Sciences in Jerusalem, with a Glowbar source, KBr beam splitter and MCT-B detector, through an aperture of $300 \mu\text{m}$ and the natural, parallel, octahedral faces of the diamond after cleaning with acid, water and ethanol (no polishing).

FIG. 3 Schematic pressure–temperature (P – T) diagram showing relevant equilibria and geothermal gradients. 91 and 92 are isochores for CO_2 with a molar volume of $21 \text{ cm}^3 \text{ mol}^{-1}$ using the equations given in refs 17, 18. The dotted area around the lines indicates the uncertainty ($21 \pm 0.4 \text{ cm}^3 \text{ mol}^{-1}$). Cross-hatched areas (from right to left) are the average current mantle adiabat³², the geotherm¹⁹ for a shield with 40 mW m^{-2} heat flux, and a subducted slab geotherm³². See text for reactions (1)–(5). Reactions (3a) and (3b) are represented by a single curve (3), as they are almost identical (similarly for reactions (5a) and (5b)). Below the intersection of reactions (5) and (3), reaction (5b) changes so that CO_2 is consumed during melting. Reaction (6) is the minimum melting curve in the CaO – MgO – SiO_2 – CO_2 system²² and is assumed to follow dolomite + coesite = liquid + CO_2 at high pressures. Reaction (4) is modified relative to reaction (3) using the P– T software²⁷. D/G represents the diamond–graphite boundary³³.



ducted sediments. High-pressure experimental data suggest that the assemblage silica + carbonate is stable to very high pressures and may yield free CO_2 by melting or decarbonation reactions. As can be seen in Fig. 3, decarbonation is less likely to occur, as it takes place at these pressures, only at rather high temperatures (for example, reaction (3) or (4)). In the simple CaO – SiO_2 – CO_2 or MgO – SiO_2 – CO_2 systems, melting releases CO_2 (refs 22, 29)



at relatively high temperatures (Fig. 3). No experimental data exist for the melting at high pressures in the mixed system CaO – MgO – SiO_2 – CO_2 , but melting temperatures of $1,300$ – $1,400^\circ\text{C}$ were estimated at 6 – 8 GPa (Fig. 3, reaction (6); see ref. 22). A cold block of carbonaceous sediments may be subducted to

depths of 220 – 270 km , where it approaches normal mantle temperatures after a storage period. During heating, the line representing reaction (6) could be crossed, and CO_2 released. Another source for C and CO_2 may be the oxidation of reduced carbon that may be present in the subjected sediments.

Although the source of the CO_2 cannot be defined at present, the work reported here gives direct evidence of both the presence of fluids at depths of 220 – 270 km and the ability of diamonds to trap and carry such fluids to the surface. If the source of the CO_2 is the degassing of melts at depth, similar events may be important as a trigger for kimberlite eruptions. If the CO_2 originated from subducted carbonaceous sediments, that implies that subduction can reinject carbonates deep into the mantle. These carbonates can be stored and later reappear in hotspot volcanism^{30,31}, supply the necessary carbon for the formation of kimberlitic and carbonatitic melts, and play a role in diamond formation. □

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The depth dependence of earthquake duration and implications for rupture mechanisms

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THE duration of rupture is a fundamental characteristic of earthquakes, and is important for understanding the mechanics of faulting^{1,2}. The complexity of the seismic source and the incoherence of the high-frequency seismic wavefield often inhibit the identification, location and timing of features in the later part of earthquake rupture. Here we sum many teleseismic records from regional seismic arrays, producing an unusually clear depiction of the earthquake source at short periods by suppressing background noise and coda generated near the receivers. The ending, as well as the beginning, of rupture is clearly identifiable for most earthquakes examined. Measurements of 130 large earthquakes show that near 100 km depth, rupture duration averages 11 s when scaled to a moment of 10^{26} dyn cm; this decreases to 5.5 s at 650 km depth. Models of faulting suggest that duration should be inversely proportional to the shear-wave velocity and the cube root of stress drop. Thus, to explain the observed twofold decrease in duration with depth, stress drops would have to increase by a factor of four, as shear velocity increases with depth by only about 20%. However, observed stress drops show no strong trend with depth^{3,4}, suggesting that the faulting process changes with depth.

We have collected teleseismic recordings of 162 earthquakes for this study, across the Northern California Seismic Network, the Southern California Seismic Network and the University of Washington Seismic Network. The networks each have several hundred short-period vertical-component seismometers that are used primarily to study regional seismicity and tectonics⁵.

From April 1980 to June 1992 there were 169 earthquakes in the distance range from 35–90° from California, larger than 1.6×10^{25} dyn cm in moment and more than 100 km in depth. The chosen distance range ensures that little distortion in the waveform results from propagation through the mantle, the moment range ensures that there is sufficient duration of rupture to measure reliably, and the depth range ensures that reflections from the surface above the earthquake (pP and sP) arrive later than the end of the direct waves.

As the aperture of each network spans several hundred kilometres and the earthquakes are 3,000–10,000 km distant, each station should see similar P-wave arrivals from a given earthquake. However, crustal structure, which is different for each station, produces a tail of scattered waves after the direct P wave that distorts and obscures the later part of each arrival.

We then isolate the component of the P arrival that is common to all stations. First, records from the stations with high noise levels or anomalously strong scattered coda are eliminated. This leaves 50–200 clean seismograms for each event. Next, these traces are aligned by eye on an easily identified feature in the first second or two of the P-wave arrival. Finally, the traces are normalized to a peak amplitude of unity and added together.

As the scattered energy is incoherent between stations, in the stacked trace it is diminished by a factor of $\sqrt{N}$ where N is the number of traces stacked. Thus, we recover the direct arrivals by cancelling the scattered coda. Although coda scattered within tens of kilometres of the source contributes to our stacked trace, its amplitude is expected to be small. The abrupt termination of most of the stacked traces confirms that near-source scattering

usually does not obscure the observation of end of the source.

The effectiveness of this procedure is demonstrated by Fig. 1a, which shows the sum of 69 short-period traces for a deep earthquake under Argentina. From the P-wave stack shown in Fig. 1a, the beginning and end of the rupture may be reliably identified at 9.5 and 15.5 s. The rupture duration is 6 s. More examples are shown in Fig. 1b.

For completeness, we note that 39 of the 169 events meeting our selection criterion were not used. P waves from 24 of these events were too near a node in the radiation pattern to allow reliable identification of the end of the rupture. Events are considered too nodal if the P radiation coefficient, which is calculated from the Harvard Moment Tensor mechanism, is less than 0.1. Two events showed two episodes of faulting, separated by 5–10 s of quiescence. Six earthquakes, all in the depth range 100–350 km, are omitted because the end of rupture is difficult to identify. Seven events did not trigger any of the three arrays,

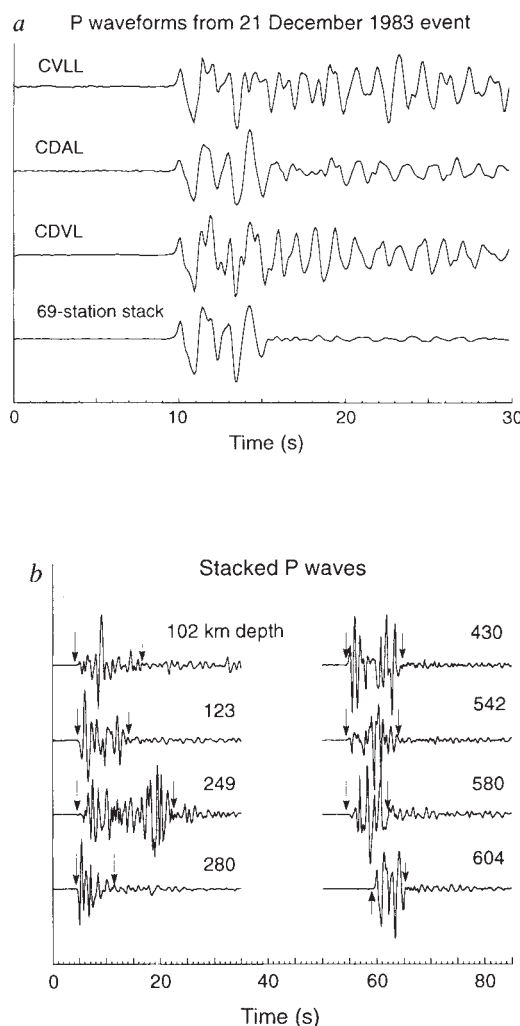


FIG. 1 a, An example of the stacking process. The top three traces are short-period vertical-component seismograms from the stations CVLL, CDAL and CDVL in the Northern California Seismic Network. The bottom trace is the sum (or stack) of 69 high-quality traces, selected from the 400 stations of the network, on the basis of low background noise, small near-surface reverberations and the absence of clipping. Note the well defined ending of the short-period P-wave radiation. b, Eight stacks of earthquakes with moments in the range $1.7\text{--}3.2 \times 10^{26}$ dyn cm. The arrows indicate the inferred start and end of each rupture. The depth of the events are given. The origin times of the traces are arbitrary.

suggesting these events produced weak and perhaps protracted short-period waves.

Figure 2 shows the durations of the remaining 130 events. Larger earthquakes have a longer duration, as expected, because the rupture length is greater. Deeper earthquakes generally rupture more rapidly than shallow and intermediate events. As earthquake duration is proportional to the linear dimension of the fault, which in turn is proportional to the cube root of moment⁶, we scale the durations to a moment of 10^{26} dyn cm and compare scaled durations with depth in Fig. 3. The tendency for the durations of the deeper events to be shorter is clear. The intermediate and shallow events show more variation in scaled duration than do the deep events.

This study contains many more duration measurements than previous studies, and the durations are measured with greater accuracy. Figure 3 indicates an average scaled duration of 11 s for events with 10^{26} dyn cm moment at 100 km depth, decreasing to 5.5 s for events near 670 km depth. It has also been shown that rise times are about twice as fast for deep earthquakes as for intermediate depth events³. Our present work shows that not only the initiation, but the entire rupture process is faster for deep earthquakes by roughly the same amount.

Previous studies, predominantly of shallow earthquakes, found average durations of 12 s (ref. 7) and 10.2 s (refs 8, 9), which are consistent with our estimates. Our durations are also generally consistent with previous duration estimates for deep earthquakes^{10–13}. The studies may differ because previous long-period estimates fit the duration of the largest long-period pulse, whereas our short-period stacks can clearly show the beginning and ending of short-period radiation from the source. The use of inaccurate Green's functions to model shallow earthquakes can make some of the previous estimates too long¹⁴. Taken together, the other studies show considerable scatter, which may result from the different methodologies used in each study.

A limitation of our data is that it samples seismic radiation in essentially only one direction from the source. If the rupture proceeds unilaterally directly towards or away from the ray path to the array, then the measured durations could be significantly shortened or lengthened, respectively. This phenomenon is termed directivity. However, inversions for the slip distributions in deep earthquakes suggest that their rupture is generally not purely unilateral¹². Moreover, we found no dependence of scaled duration on subduction zone. Accordingly, it appears that our measured durations are not severely affected by directivity.

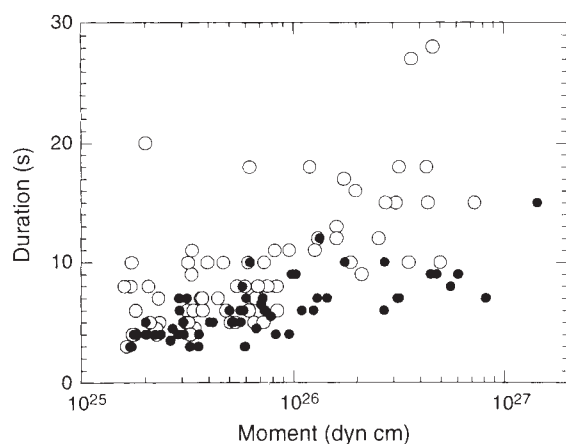


FIG. 2 Seismic moment is plotted against the earthquake duration measured from short-period stacks, as illustrated in Fig. 1. Duration increases as the cube root of moment, as predicted by source scaling relations⁶. Moments are from the Harvard Centroid Moment Tensor catalogue²². ○, Depth < 350 km; ●, depth > 350 km.

The observation of high-frequency seismic radiation throughout the entire rupture duration coupled with the good agreement with more broad-band duration estimates suggests that significant slow afterslip is not a general feature of moderately large earthquakes. Similarly, the sharp initiation of each event precludes more than about 1 to 2 s of accelerating slow slip at the start. Our sensitivity is limited to frequencies between 0.2 and 2 Hz, however.

We would expect some decrease in the duration of earthquakes with depth solely because the shear velocity increases with depth, leading to faster expansion of the rupture front. To explore such effects, consider a circular crack model. The seismic moment is given by

$$M_0 = \mu \pi r^2 D$$

where μ is the shear modulus, D is the average fault slip, and r is the radius of the fault area. The static stress drop during an earthquake is proportional to the displacement divided by a fault dimension⁶:

$$\Delta\sigma \propto \frac{\mu \pi D}{r} = \frac{M_0}{r^3}$$

Therefore

$$r \propto \left(\frac{M_0}{\Delta\sigma} \right)^{1/3}$$

and duration τ

$$\tau \propto \frac{r}{v_s} \propto \frac{M_0^{1/3}}{v_s \Delta\sigma^{1/3}}$$

assuming that rupture velocity is proportional to the shear wave velocity v_s . Thus, scaled durations would be expected to follow

$$\frac{\tau}{M_0^{1/3}} \propto \frac{1}{v_s \Delta\sigma^{1/3}}$$

Therefore, for this model to explain the observed decrease in scaled durations with depth of a factor of two from 100 to 670 km, stress drops would have to increase with depth by a factor of four as v_s increases by only 20% over this depth range. Observed stress drops, however, show no such dramatic increase according to a review of eight studies⁴, as well as ref. 3, which is discussed below.

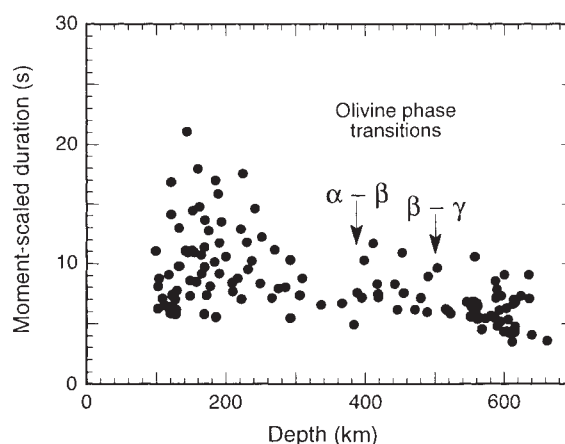


FIG. 3 Moment-scaled duration plotted against depth. The scaled duration is obtained by dividing the duration shown in Fig. 2 by the cube root of moment, normalized to 10^{26} dyn cm. The decrease in duration with depth is greater than that expected from source scaling relations, which predict that duration should be proportional to the inverse of the shear-wave velocity.

Stress drops are difficult to measure and have been estimated by several methods, including using durations measured from pulse widths, as mentioned above^{13,15}. Stress drop and duration, although related, are fundamentally different physical properties. It is possible for an event to have both high stress drop and long duration or low stress drop and short duration, depending on the detailed shape of the source time function, or equivalently, the source spectrum. One problem with using duration to estimate stress drops, besides the measurement difficulty mentioned above, is that it uses essentially only one part of the source spectrum, the corner frequency. An independent way of measuring stress drop that uses the entire bandwidth of the source spectrum was applied to 68 intermediate and deep earthquakes³. In this work, seismically-radiated energies were estimated from the squared velocity-source spectrum of broadband teleseismic body waves recorded by the Global Digital Seismic Network (GDSN). From the ratio of energy to moment, an Orowan stress drop can be calculated¹⁶. These stress drops were found to be constant with depth³.

Therefore, our observation of durations decreasing by a factor of two, while the Orowan stress drop remains constant with depth, requires that some element of rupture changes with depth be incorporated into the above model. Why might earthquake rise time and duration depend on depth? The fault geometry could become less elongated and more equi-dimensional with depth. Alternatively, faulting at shallower depths may be more episodic or non-steady, perhaps because of the greater heterogeneity in temperature and composition that is present nearer the surface of the Earth. These two explanations may be related; elongated ruptures may be more intermittent, whereas equi-dimensional faulting might spread out more smoothly as the rupture develops. It is also possible that the rupture velocity is increasing faster with depth than the shear-wave velocity. The observed decrease in duration with depth, together with a constant stress drop, suggests a change in the shape of the source spectrum. Specifically, intermediate depth events would need to have more high-frequency energy (radiation at periods shorter than the earthquake duration) and deep events less such energy.

These rupture properties, in turn, may be influenced by a transition from frictional faulting to transformational faulting at intermediate depths^{17,18}. They could also be influenced by the transition from α -olivine to β -phase near 390 km depth, the transition from β -phase to γ -phase near 500 km depth, or other phase transitions within subducting slabs¹⁹. The depths above are appropriate for temperatures within a cold slab²⁰, and the α to β conversion depth in slabs is supported by seismic measurements²¹. These depths also correspond to the minima in the frequency of earthquakes visible in Fig. 3.

The depth dependence of the durations shown in Fig. 3 suggests that phase transitions may be influential. Events in the α -olivine stability field show the largest variation in duration. Events in the β -phase region have more moderate variations, and the events in the γ -phase region, which are the shortest duration events, show the least variation in duration. □

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Magnetite formation by a sulphate-reducing bacterium

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BACTERIAL production of magnetite (Fe_3O_4)¹ makes an important contribution to iron biomineralization and remanent magnetization of sediments^{2,3}. Accurate magnetostratigraphy, reconstruction of the Earth's past magnetic-field behaviour and extraction of environmental information from the geomagnetic record depend on an understanding of the conditions under which bacterial magnetite is formed. In aquatic sediments, the process is thought to be restricted to a zone between the levels at which nitrate and iron reduction occur⁴. In sulphate-reducing habitats, deeper in the sediment, the presence of H_2S reduces iron oxyhydroxides to iron sulphides^{5,6}. Thus magnetite would not be expected to form under such reducing conditions^{5,7}. We report here the isolation and pure culture of a dissimilatory sulphate-reducing bacterium, designated RS-1, which can synthesize intracellular magnetite particles. RS-1 is a freshwater anaerobe which is also capable of extracellular iron sulphide precipitation. This isolate illustrates the wider metabolic diversity of magnetic bacteria and suggests the presence of a novel mechanism of magnetic biomineralization. The discovery of such bacteria may also explain why large quantities of magnetite have been observed in sulphate-rich, oil-bearing, sedimentary deposits^{8–11}. In addition, these results significantly enlarge the environments in which biogenic magnetite may be expected to occur and have important implications regarding the evolution of the ability to synthesize magnetite.

Freshwater sulphide-rich mud was collected from a waterway near Kamen river, Wakayama prefecture, in western Japan. Samples were allowed to enrich for 2 weeks in 1-l glass containers. Magnetic bacteria were not observed in water from these enrichments. Samples of the water were inoculated anaerobically into a modified isolation medium¹². One week later, a Gram-negative magnetic bacterium, designated RS-1, was isolated in pure culture by restreaking single colonies onto isolation-medium agar plates. Cells were 3–5 μm in length with a diameter of 0.9–1.5 μm , with a helicoid to rod shaped morphology and a single polar flagellum (Fig. 1a). The guanine plus cytosine content of purified genomic DNA was $65.8 \pm 0.2\%$ per cent, as determined by HPLC. Additional morphological characteristics differed from those of previously cultured magnetic bacteria^{12–18}. Although RS-1 cells oriented and swam along magnetic-field lines (magnetotaxis) (Fig. 1b), they showed stronger anaerotaxis and accumulated at an anaerobic zone in the centre of a slide preparation with artificial magnetic fields. Individual cells could reverse their swimming direction without turning round. The ratio of magnetic north to south seeking cells was 1:1 in a population of cultured cells.

Although RS-1 was catalase positive and oxidase negative, it could not be cultured in the presence of oxygen and was a strict anaerobe. RS-1 could only grow in the presence of sulphate:

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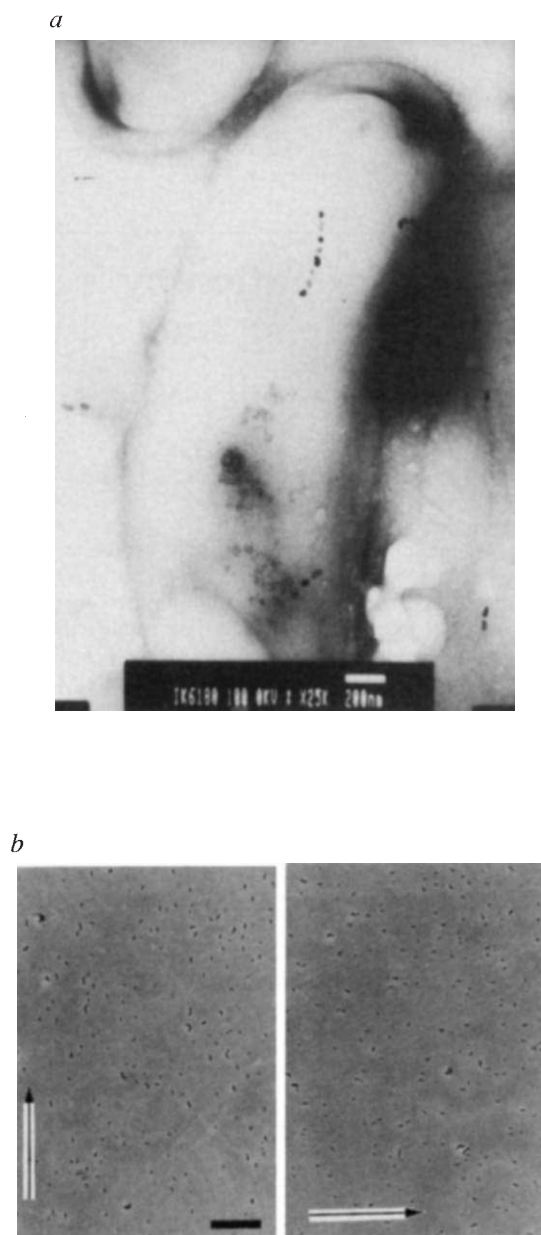


FIG. 1 a, Transmission electron micrograph (TEM) of RS-1 grown anaerobically in a modified isolation medium. Scale bar, 200 nm. b, Phase-contrast photomicrograph showing orientation of living RS-1 cells in artificial magnetic fields. Arrow represents direction of magnetic axis (bar, 50 μ m).

METHODS. Cells were negatively stained with 1% phosphotungstic acid and observed by TEM (JEOL 1200EX). Growth medium consisted of 2 ml mineral solution, 2 ml ferric quinate, 0.2 g potassium dihydrogen phosphate, 0.06 g ammonium chloride, 0.5 g malate, 0.5 g sodium sulphate $10 \cdot \text{H}_2\text{O}$, and 0.3 g l^{-1} yeast extract (Difco) in 1 l of distilled water. The medium was adjusted to pH 7.0 with NaOH solution before sterilization (120°C , 2 atm., 10 min). The mineral solution was modified from Wolf's mineral solution³³: all sulphates were exchanged with chlorides and nickel was added. Composition was as follows per l distilled water: 1.8 g sodium nitriloacetate, 2.5 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.6 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1.0 g NaCl, 0.1 g $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 0.1 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.13 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01 g ZnCl_2 , 0.01 g $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01 g H_3BO_3 , 0.01 g NaMoO_4 , $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$. For anaerobic conditions, gas in the culture headspace was replaced with argon gas. Absence of oxygen was checked by gas chromatography. All experiments used glass vials with air-tight butyl rubber with aluminium caps. H_2S was detected in the stationary-phase culture of RS-1 with lead acetate paper³⁴. The black precipitate (iron sulphides) was dissolved in 5 M HCl, and qualitatively analysed using the ferrozine³⁵ and CuS methods³⁶.

if sulphate was omitted from the growth medium, growth was extremely inhibited or did not occur. Growth was not triggered by a low redox potential, and it was not necessary to add redox reagents such as sodium thioglycolate or ascorbate to the medium. RS-1 could utilize pyruvate, lactate, ethanol, malate and fumarate but not acetate, using inorganic sulphate as the terminal electron acceptor. H_2S production occurred only in the presence of inorganic sulphate under anaerobic conditions. In a chemically defined medium, which did not contain sulphate, H_2S production was not observed after the addition of sulphur-containing amino acids or yeast extract. Little or no growth occurred after addition of these compounds at concentrations of 0.05 – 0.1 g l^{-1} . RS-1 cultures containing sulphate turned black due to the generation of insoluble iron sulphide, a phenomenon characteristic of sulphate-reducing bacteria. The culture became strongly reduced after cell growth (below -100 mV Eh). RS-1 cells also showed hydrogenase activity. Growth was inhibited in the presence of sodium nitrate (over 0.3 g l^{-1}), and not enhanced by the addition of low levels of nitrate (0.01 – 0.05 g l^{-1}). Nitrate did not support growth as an electron acceptor. From the above observations we concluded that RS-1 is a dissimilatory sulphate-reducing bacterium.

Although intracellular synthesis of iron sulphides has been observed in sulphate-reducing bacteria¹⁹ and magnetic bacteria^{20,21}, RS-1 represents the first sulphate-reducing bacterium capable of producing the iron oxide magnetite (Fig. 2). Microbiological formation of magnetite can occur in the presence of molecular oxygen in *Magnetospirillum magnetotacticum* strain MS-1, which can use oxygen or nitrate as the terminal electron acceptor²². Nitrate is also used as a terminal electron acceptor by the oxygen-tolerant strain *Magnetospirillum* AMB-1¹². The dissimilatory iron-reducing bacterium *Geobacter metallireducens* strain GS-15²³ can synthesize extracellular magnetite anaerobically using ferric iron as the terminal electron acceptor. Nitrous oxide is used as the terminal electron acceptor by the marine vibroid magnetic bacterium strain MV-1 during anaerobic production of magnetite¹³. Our results indicate that sulphate-reducing bacteria can also contribute to the production of magnetite under strictly anaerobic conditions. Moreover, the sedimentary zone in which magnetite may be found is now significantly extended to the deeper levels at which sulphate reduction occurs^{24,25}. In these sulphate-reducing zones, iron hydroxides are converted to iron sulphides by H_2S ^{5,7}. The production of magnetite by RS-1 under reducing conditions is surprising and suggests that the mechanism of magnetite formation may be different from that which occurs in nitrate-reducing magnetic bacteria.

RS-1 is also capable of extracellular precipitation of magnetic iron sulphides. Thus we conclude that a single species of sulphate-reducing bacteria can contribute to iron biomineralization and sedimentary magnetization using oxygen (Fe_3O_4) as well as sulphur (FeS and so on), and can contribute to the formation of single-domain magnetite in sulphur-rich (sulphate-reducing) anaerobic sediments.

Oil-producing wells are anaerobic sulphate-rich environments in which very large quantities of fine-grained magnetite have been observed^{8,9}. It has been suggested that the magnetite is of biological origin¹⁰, but no evidence for this has been described. Sulphate-reducing bacteria occur in such habitats¹¹. The discovery of a sulphate-reducing bacterium capable of synthesizing fine-grained magnetite may provide one explanation for the existence of these magnetite sludges.

In addition to bacteria, certain invertebrates can synthesize magnetite for use as a tooth hardener; magnetite particles have also been observed in many higher organisms where they appear to act as navigation aids²⁶. Despite the biological importance of these phenomena, little information is available on the evolution of magnetite synthesis. We have begun to analyse the genetic basis of magnetite synthesis in bacteria²⁷ and to determine the evolutionary relationships among cultured magnetite-containing

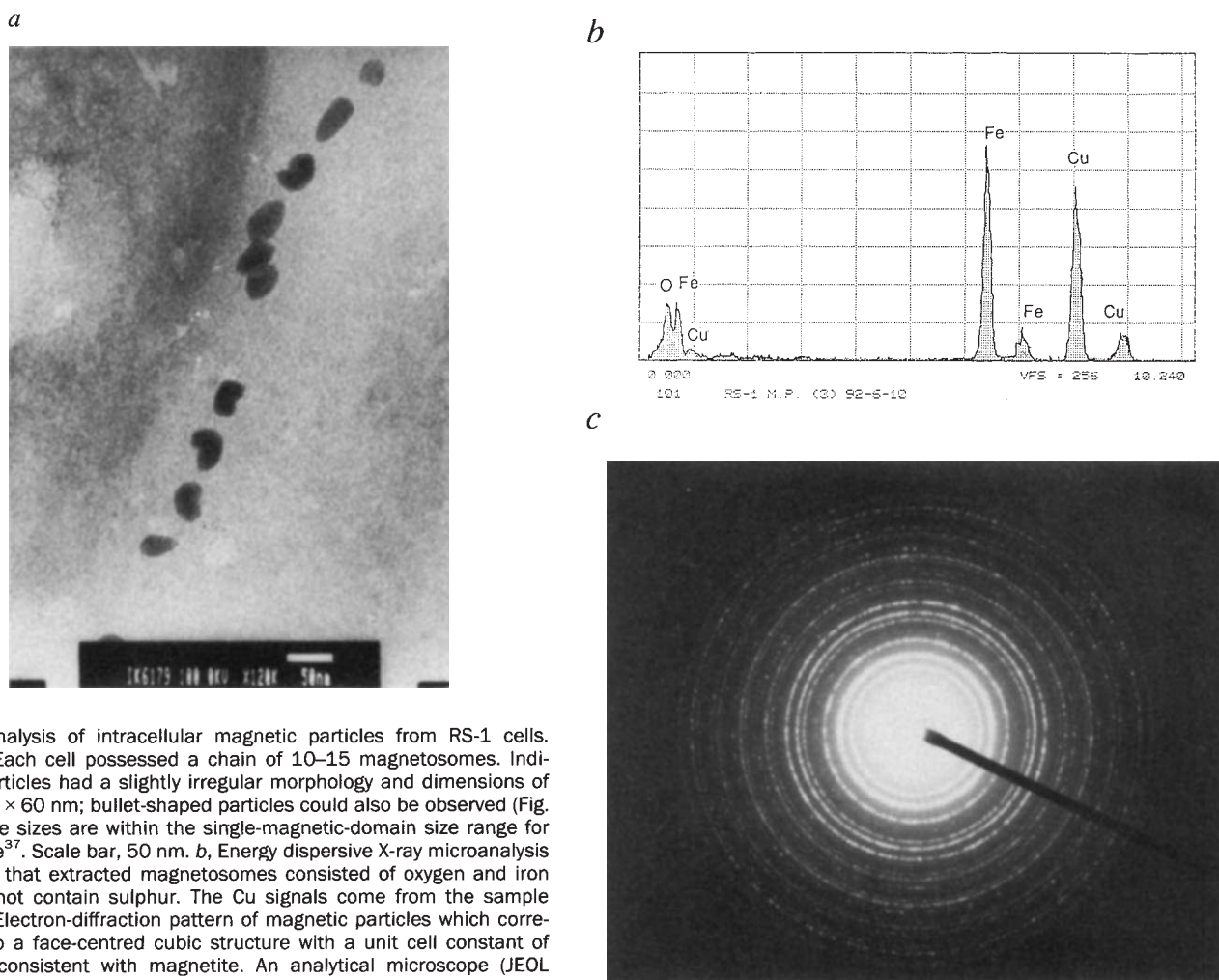


FIG. 2 Analysis of intracellular magnetic particles from RS-1 cells. *a*, TEM. Each cell possessed a chain of 10–15 magnetosomes. Individual particles had a slightly irregular morphology and dimensions of $\sim 40 \times 40 \times 60$ nm; bullet-shaped particles could also be observed (Fig. 2*a*). These sizes are within the single-magnetic-domain size range for magnetite³⁷. Scale bar, 50 nm. *b*, Energy dispersive X-ray microanalysis indicated that extracted magnetosomes consisted of oxygen and iron and did not contain sulphur. The Cu signals come from the sample grids. *c*, Electron-diffraction pattern of magnetic particles which corresponds to a face-centred cubic structure with a unit cell constant of 8.3358, consistent with magnetite. An analytical microscope (JEOL JEM-2000FX) was used.

bacteria²⁸. Phylogenetic analyses of magnetic bacteria indicate that the ability to synthesize magnetite is confined to the α subdivision of the proteobacteria^{16,28–31}. Preliminary analysis of the small subunit ribosomal RNA from RS-1, suggests that this bacterium is not associated with the proteobacteria (manuscript in preparation). This indicates a polyphyletic origin for intracellular magnetite synthesis. RS-1 magnetosomes also have a different morphology (irregular bullet-shaped) from previously cultured magnetic bacteria. Iron sulphide-containing magnetic bacteria are associated with the δ subdivision, indicating a polyphyletic origin for magnetotaxis within the proteobacteria³¹.

These findings have important implications, regarding the recent debate about iron and the origin of life³², because both iron oxide minerals and iron sulphides are produced by RS-1. A thorough investigation into the metabolic relationship between sulphate reduction, magnetite synthesis and iron sulphide precipitation in RS-1 may provide information about the origin of energy metabolism in primitive bacteria. □

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Inverse-dynamics model eye movement control by Purkinje cells in the cerebellum

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MANY lines of evidence suggest that the cerebellum is involved in motor control¹. But what features of these movements are encoded by cerebellar neurons? For slow-tracking eye movements, the activity of Purkinje cells in the ventral paraflocculus of the cerebellum is known to be correlated with eye velocity²⁻⁵ and acceleration⁵. Here we show that the complex temporal pattern of the firing frequency that occurs during the ocular following response elicited by movements of a large visual scene⁶⁻⁸ can be reconstructed by an inverse-dynamics representation, which uses the position,

velocity and acceleration of eye movements. Further analysis reveals that the velocity and acceleration components can provide appropriate dynamic drive signals to ocular motor neurons, whereas the position component often has the wrong polarity. We conclude that these Purkinje cells primarily contribute dynamic command signals.

Figure 1 shows the sample simple spike activity of a Purkinje cell (P cell) in the left ventral paraflocculus of a monkey together with the ocular following response to 65 presentations of a 160 deg s⁻¹ downward test ramp of a large random-dot pattern. Responses were aligned with stimulus onset (time 0). From top to bottom, the ensemble average spike response over the 65 trials of this P cell, eye acceleration, velocity and position, and stimulus velocity are shown. The firing rate of this neuron becomes significantly different from the baseline rate 51 ms after the onset of stimulus motion. In the data shown here, the eyes began moving several milliseconds after the onset of the neural responses. To analyse the information represented in the activity of the P cell, a temporal pattern of ensemble average firing frequency of the P cell was reconstructed by the inverse-dynamics representation method^{9,11} as follows:

$$f(t - \Delta) = a \cdot e''(t) + b \cdot e'(t) + c \cdot e(t) + d \quad (1)$$

where $f(t)$, $e''(t)$, $e'(t)$, $e(t)$, Δ are the firing frequency at time t , the eye acceleration, velocity and position at time t , and the

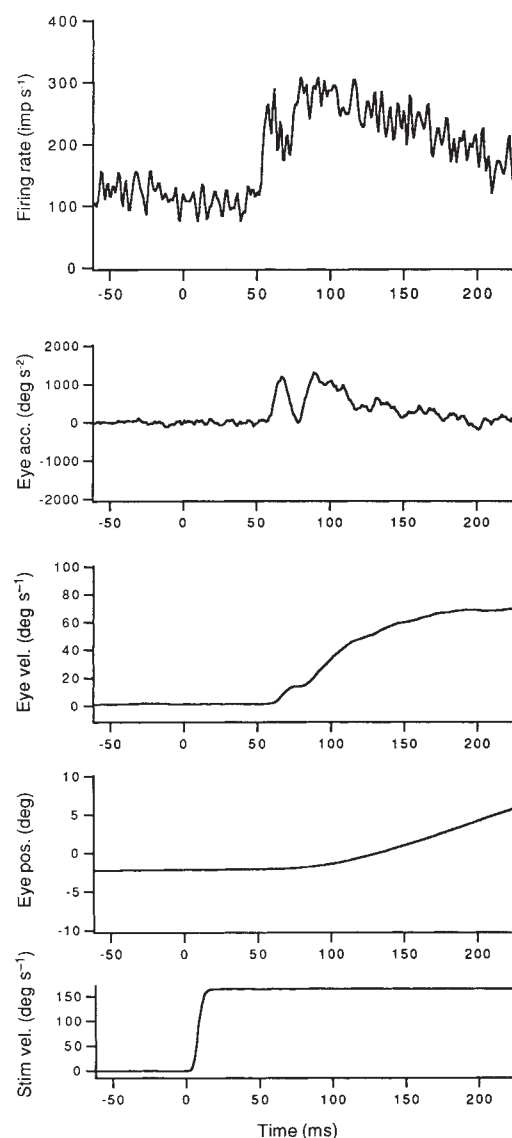


FIG. 1 Simple spike activity of a P cell in the left ventral paraflocculus and the ocular following responses to 65 presentations of a 160 deg s⁻¹ downward test ramp. Responses are aligned at a stimulus onset (Time 0). From top to bottom: ensemble average firing frequencies, averaged vertical eye acceleration, velocity and position, and averaged stimulus velocity profiles. Upward direction in the figure means downward eye or stimulus movements. All eye-movement data were filtered with a 6-pole Bessel low-pass filter (cut-off, 100 Hz). Latency was 51 ms for unit activity.

METHODS. The animals (*Macaca fuscata*) had been previously trained to fixate a small target spot to obtain a fluid reward. Under Nembutal anaesthesia and aseptic conditions, each monkey was implanted with a cylinder for microelectrode recording, and fitted with a head holder that allowed the head to be fixed in the standard stereotaxic position during the experiments. Scleral search coils were implanted for measuring eye movements²¹. The animals faced a translucent tangent screen (85° × 85° at a distance of 235 mm) on which moving random-dot patterns could be back-projected. The visual stimulus started to move 150 ms after the end of a centering saccade with eight directions at 10–160 deg s⁻¹. Each visual stimulus ramp lasted 250 ms. Voltage signals separately encoding the horizontal and vertical components of eye position, eye velocity, and mirror (stimulus) velocity were digitized to a resolution of 12 bits, sampling at 500 Hz⁸. The acceleration profiles were obtained by digital differentiation of eye velocity profiles after averaging. A time-amplitude window discriminator was used to record spike occurrences with a time resolution of 1 ms.

TABLE 1 Significance of each component in the reconstructed firing frequency

	$P < 0.005$	$0.005 \leq P \leq 0.05$	$P > 0.05$
Acceleration component	18	1	0
Velocity component	19	0	0
Position component	16	0	3
Bias component	19	0	0

P values of t -tests are for the null hypothesis that a coefficient of each component is zero, and indicate the probability that the model fitting is performed well when a particular component is dropped. Numbers denote the number of cells.

time delay between firing frequency and movement, respectively. Four coefficients (a, b, c, d) and the time delay (Δ) were estimated to minimize the squared error between the average and reconstructed firing frequencies. This inverse-dynamics equation looks similar to the forward-dynamics model of the eye plant, which predicts the movement from the motor command, but is very different from and actually opposes it in the direction of information flow^{12,13}.

If we assume, for simplicity, the final motor neuron command $m(t)$ to be the weighted sum of the firing $f_i(t)$ of the i th P cell weighted by w_i , and the firing $g_j(t)$ of the j th neuron weighted by p_j in another brain region, then we can derive the following second-order forward-dynamics equation with the acceleration, velocity and position coefficients M, B, K ^{14,15}:

$$M \cdot e''(t) + B \cdot e'(t) + K \cdot e(t) = m(t - \Delta_m) = \sum_{i=1}^N w_i \cdot f_i(t - \Delta_i) + \sum_{j=1}^L p_j \cdot g_j(t - \Delta_j) \quad (2)$$

This forward-dynamics equation requires that all casual inputs

FIG. 2 Reconstruction of temporal pattern of firing frequency of a P cell by inverse-dynamics representation using the position, velocity and acceleration of the eye movements. The temporal pattern of the ensemble average firing frequency of the P cell in Fig. 1 was reconstructed, using the data at stimulus velocities of 10, 20, 40, 80 and 160 deg s⁻¹ together. The squared error for model fitting reached minimum at a delay of 8 ms. The determination coefficient was 0.84. The acceleration coefficient was 0.11 spikes s⁻¹ per deg s⁻², the velocity coefficient was 2.7 spikes s⁻¹ per deg s⁻¹, the position coefficient was -20.4 spikes s⁻¹ deg⁻¹. For superimposition, eye movement profiles were shifted by the delay time. A, Reconstructed firing frequency profile of a P cell superimposed on the raw firing frequency profile, and the reconstructed acceleration, velocity and position components. The stimulus velocity was 160 deg s⁻¹. Reconstructed firing frequency profile (red line), raw firing frequency profile (black dotted line), acceleration component (purple line), velocity component (green line), position component (blue line). B, Reconstruction of temporal pattern of firing frequency of the P cell at different stimulus velocities: a, 10 deg s⁻¹; b, 20 deg s⁻¹; c, 40 deg s⁻¹; d, 80 deg s⁻¹. Reconstructed firing frequency profile is represented by a red line, and the raw firing frequency profile by a black dotted line.

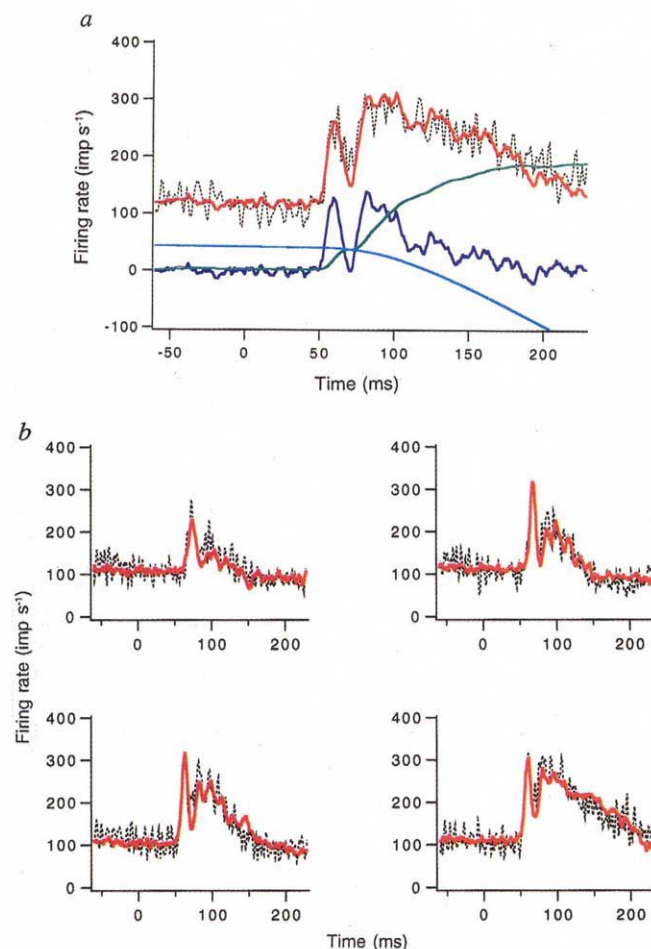
METHODS. The temporal pattern of ensemble average firing frequency of a P cell was reconstructed as a time series from linear combinations of the eye position, velocity and acceleration after a fixed delay time for the firing frequency, and a bias term. Four coefficients and the time delay were estimated to minimize the squared estimation error. The calculation was done using Mathematica on a Sun Sparc Station.

are to the eye plant and can be used only for the final common path^{14,16} not for a single P cell: that is, movement cannot be predicted from single P cell firing. But the inverse-dynamics represented in equation (1) can be used for a single P cell if its output contributes to the final motor command. Furthermore, in this inverse-dynamics representation any coefficients (a, b, c) would be negative if activities of other cells or regions compensate for its negative contribution to provide the final and stable motor neuron command: that is, the single P cell firing could be reconstructed from the movement.

The response properties of the P cell whose 160 deg s⁻¹ response is shown in Fig. 1 were next studied at five different stimulus velocities (10, 20, 40, 80, 160 deg s⁻¹). Parameter estimation was made for data from all five velocities together. The estimation error was minimal at 8 ms delay. The determination coefficient¹⁷, which is the square of the correlation coefficient (r^2), was 0.84, indicating that the simple linear inverse-dynamics representation can satisfactorily predict complex time courses of P cell firing.

Figure 2A shows the superimposed traces of the raw firing frequency (black dotted line) and the reconstructed firing frequency (red line) of the P cell in Fig. 1 using these parameters, together with the contribution of each component of the eye movements to the reconstructed firing frequency. Although the acceleration coefficient (a) was small (0.11 spikes s⁻¹ per deg s⁻²), its component ($a \cdot e''(t)$; purple line) was predominant in the initial phase of the response and the velocity component ($b \cdot e'(t)$; green line) was dominant in most of the rest. In the later phase, the acceleration component decreased and the position ($c \cdot e(t)$; blue line) component increased. The position component, however, was of reversed sign compared with the direction of the movements.

The successful reconstruction of the firing frequency at differ-



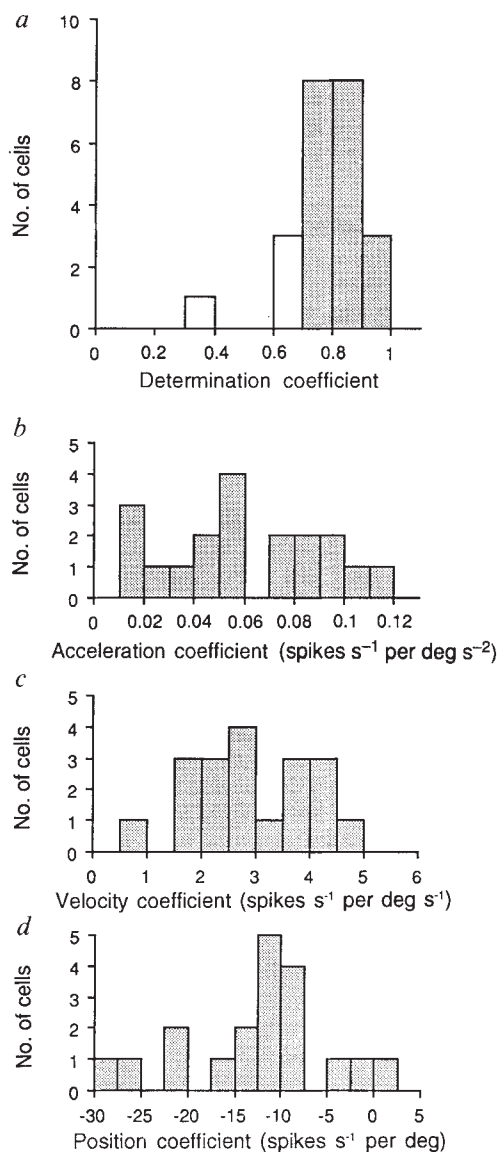


FIG. 3 The distribution of various parameters in model fitting. *a*, Determination coefficients of 23 P cells at the stimulus velocity of 80 or 160 deg s⁻¹. Shaded bars ($n=19$) indicates data whose determination coefficients were more than 0.7. The subsequent statistical analysis was performed on these 19 well-fitted P cells. *b*, Acceleration coefficients of 19 P cells. The mean ($\pm$ s.d.) was 0.061 ($\pm$ 0.032) spikes s⁻¹ per deg s⁻². *c*, Velocity coefficients; mean ($\pm$ s.d.) was 2.9 ($\pm$ 1.0) spikes s⁻¹ per deg s⁻¹. *d*, Position coefficients; mean ($\pm$ s.d.) was -12.6 ($\pm$ 7.8) spikes s⁻¹ deg⁻¹.

ent stimulus velocities by a single set of parameters (Fig. 2*A*: 160 deg s⁻¹; Fig. 2*B*: *a*, 10 deg s⁻¹; *b*, 20 deg s⁻¹; *c*, 40 deg s⁻¹; *d*, 80 deg s⁻¹) indicates the generality of the model.

We studied 43 P cells in the left ventral paraflocculus of two monkeys at a stimulus velocity of 80 or 160 deg s⁻¹ (ref. 8). Of these P cells, the same analysis was executed on 23 P cells whose activities could be recorded during the 252 ms after stimulus onset on more than 25 trials without contamination of saccades. The determination coefficient was more than 0.7 in 19 cases (Fig. 3*a*). The parameters of the 19 well fitted P cells were analysed statistically. The mean delay was 7.1 ms ($\pm$ 2.8 ms s.d.), which was near the latency of electrical-stimulation-evoked eye movements⁸. This satisfies a basic prerequisite for the firing frequency to represent the motor command. The distribution of each coefficient is shown in Fig. 3*b-d*. The acceleration coefficient had a value of 0.061 ± 0.032 spikes s⁻¹ per deg s⁻² (mean $\pm$ s.d.). The velocity coefficient was 2.9 ± 1.0 spikes s⁻¹ per deg s⁻¹ (mean $\pm$ s.d.). The position coefficient was -12.6 ± 7.8 spikes s⁻¹ deg⁻¹ (mean $\pm$ s.d.). The significance of each component was tested by *t*-tests for the null hypothesis that each coefficient is zero; the results are shown in Table 1. The *P* values for each coefficient were less than 0.005 in most cases, indicating that all components were significant in contributing to the firing frequency. Further, we tested whether the firing frequency could be well fitted by dropping any of the components. The results were that the determination coefficients (r^2) for the reduced models were significantly smaller (mean was 0.73, 0.55 or 0.71 for dropping $a \cdot e'$, $b \cdot e'$ or $c \cdot e$, respectively) than the three component model (mean 0.82), indicating that all the components were necessary to represent the firing frequency.

To compare each component pertaining to P cells with its motor neuron equivalent we examined the ratios of the coefficients. The mean ratio of acceleration coefficient to velocity coefficient (b/a) of the P cells was $72.1 (\pm 72.4 \text{ s.d.})$, which was close to that of motor neurons at 67.4 (ref. 15). On the other hand, whereas the mean ratio of acceleration coefficient to position coefficient ($c/a = -294.5 (\pm 261.8 \text{ s.d.})$) of P cells was comparable in size to that of motor neurons (344.8; ref. 15), it was different and had a reversed sign, suggesting that there are other inputs to motor neurons that effectively cancel these inappropriate position signals from the ventral paraflocculus, with the net effect that these P cells contribute to the dynamic (velocity and acceleration) rather than the static (position) control of eye movements. Although the mean ratio of acceleration and velocity coefficients of P cells is close to that of motor neurons, the mean ratio of visual mossy fibre inputs to P cells is only $\sim 50\%$ (unpublished observations): that is, although the output from P cells may constitute the dynamic part of the final motor command, their inputs cannot. The inverse-dynamics problem could be solved at multiple sites in the brain, but our results support the hypothesis that the cerebellum may represent a major site for inverse-dynamics modelling of controlled movements^{13,18,20}. This computational hypothesis could be rigorously tested by applying our analysis to different sites in the brain whose activities are related to the ocular following response. □

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Different roles of $\alpha\beta$ and $\gamma\delta$ T cells in immunity against an intracellular bacterial pathogen

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SEVERAL bacterial pathogens of medical importance are able to persist and replicate inside host mononuclear phagocytes. Protective immunity depends on specific T lymphocytes that induce granulomatous lesions at the sites of bacterial multiplication^{1,2}. *Listeria monocytogenes* is an intracellular pathogen that replicates inside mononuclear phagocytes and hepatocytes of mice¹⁻⁴. Invasion from the phagosomal compartment into the cytoplasmic compartment is the principal mechanism of intracellular survival⁵. Early in infection, resistance against *L. monocytogenes* is mediated by polymorphonuclear phagocytes which destroy infected liver cells, followed by natural killer cells which activate macrophages by means of interferon- γ (refs 6, 7). A specific immune response by T cells then

develops which leads to sterile eradication of the microbes^{1,2,8}. T cells are also responsible for the highly effective protection in vaccinated mice against secondary infections^{1,2}. Although the role of $\alpha\beta$ T cells has been demonstrated in these immune responses, that of $\gamma\delta$ T cells is unclear^{2,9,10}. Here we use mice that selectively lack either $\alpha\beta$ or $\gamma\delta$ T cells as a result of targeted germ-line mutations in their T-cell receptor genes^{11,12} to investigate the relative roles of these T-cell populations during experimental infection with *L. monocytogenes*. We find that in primary listeriosis, either $\alpha\beta$ or $\gamma\delta$ T cells are sufficient for early protection. Resistance to secondary infection is mediated mainly by $\alpha\beta$ T cells but also involves $\gamma\delta$ T cells. Thus $\alpha\beta$ T-cell-deficient mice can be rendered partially resistant by vaccination, and $\gamma\delta$ T cells are shown to be responsible for this protective effect. In infected $\gamma\delta$ T-cell-deficient mice we noticed the appearance of unusual liver lesions, indicating that $\gamma\delta$ T cells have a unique regulatory role in this bacterial infection.

We previously reported the construction and initial characterization of T-cell antigen receptor (TCR)- α or TCR- β mutant mice (lacking $\alpha\beta$ T cells) and TCR- δ mutants (lacking $\gamma\delta$ T cells)^{11,12}. These studies suggested the presence of a normal $\alpha\beta$ T-cell repertoire in the periphery of the $\gamma\delta$ T-cell-deficient mice¹² and of apparently normal $\gamma\delta$ T cells in the periphery of the $\alpha\beta$ T-cell-deficient mice¹¹. Thus, we should be able to deduce whether the roles of $\alpha\beta$ or $\gamma\delta$ T cells in protection against the intracellular pathogen *L. monocytogenes* are essential and/or sufficient by analysing the mutant mice infected by this pathogen.

TCR- α , TCR- β and TCR- δ mutants as well as heterozygous littermates were intravenously infected with *L. monocytogenes* and the dose giving 50% lethality (LD₅₀) was determined as $\leq 1 \times 10^4$ (data not shown). Subsequently, TCR mutants were infected with sublethal doses of *L. monocytogenes* and colony-forming units (CFU) in spleens were determined after several

TABLE 1 Primary listeriosis in TCR- β , TCR- α , TCR- δ and TCR- $\beta \times \delta$ mutant mice*

Experiment (group)	Type of mice	No. of mice	Inoculum (in log ₁₀)	Day of testing	CFU per spleen (in log ₁₀)†	Significant difference in CFU‡
1(1)	TCR- $\beta^{-/-}$	5	3.4	8	3.3 (2.7–4.6)	vs 1(2)
1(2)	TCR- $\beta^{-/-}$ + anti-TCR- δ	5	3.4	8	5.1 (4.3–6.0)	vs 1(1), vs 1(3)
1(3)	Control	5	3.4	8	3.3 (<2–4.0)	vs 1(2)
2(1)	TCR- $\alpha^{-/-}$	5	3.4	7	4.0 (3.5–4.4)	vs 2(2)
2(2)	TCR- $\alpha^{-/-}$ + anti-TCR- δ	5	3.4	7	5.4 (4.9–5.8)	vs 2(1), vs 2(3)
2(3)	Control	5	3.4	7	3.7 (3.3–4.3)	vs 2(2)
3(1)	TCR- $\delta^{-/-}$	4	3.7	2	5.0 (4.8–5.2)	NS§
3(2)	Control	4	3.7	2	5.2 (5.1–5.3)	NS
4(1)	TCR- $\delta^{-/-}$	4	3.7	9	2.8 (2.0–3.7)	NS
4(2)	Control	4	3.7	9	3.0 (2.3–3.7)	NS
5(1)	TCR- $\delta^{-/-}$	4	5.0	2	7.2 (5.4–7.5)	NS
5(2)	Control	4	5.0	2	6.6 (6.2–7.6)	NS
6(1)	TCR- $\beta^{-/-}$	5	3.4	7	2.3 (<2–3.7)	vs 6(2)
6(2)	RAG-1 ^{-/-}	4	3.4	7	5.9 (<2–6.4)	vs 6(1), vs 6(3)
6(3)	Control	5	3.4	7	2.3 (<2–3.7)	vs 6(2)
7(1)	TCR- $\beta^{-/-}$	5	3.3	8	3.7 (3.4–3.9)	vs 7(2)
7(2)	TCR- $\beta \times \delta^{-/-}$	5	3.3	8	4.8 (3.6–5.1)	vs 7(1), vs 7(3)
7(3)	Control	5	3.3	8	3.2 (<2–3.5)	vs 7(2)
8(1)	TCR- $\beta^{-/-}$	4	3.0	20	<2 (<2–<2)	NS
8(2)	TCR- $\delta^{-/-}$	4	3.0	20	<2 (<2–<2)	NS
8(3)	Control	4	3.0	20	<2 (<2–<2)	NS
9(1)	TCR- $\beta^{-/-}$	5	3.7	21	2.5 (<2–3.2)	vs 9(3), vs 9(4)
9(2)	TCR- $\delta^{-/-}$	5	3.7	21	<2 (<2–<2)	vs 9(3), vs 9(4)
9(3)	TCR- $\beta \times \delta^{-/-}$	4	3.7	21	5.5 (5.1–6.2)	vs 9(1), vs 9(2), vs 9(5)
9(4)	RAG-1 ^{-/-}	4	3.7	21	5.4 (5.0–6.3)	vs 9(1), vs 9(2), vs 9(5)
9(5)	Control	5	3.7	21	<2 (<2–<2)	vs 9(3), vs 9(4)

* Mice were infected with a sublethal dose of *L. monocytogenes* and on days 2–21 thereafter, colony-forming units (CFU) in spleens were determined by plating serial dilutions of organ homogenate on trypticase soy plates³. Some groups of mice received 0.5 mg GL3 antibody intraperitoneally 2 days before infection. All experiments were done with >10-week-old mice of (129 $\times$ C57BL/6) or (129 $\times$ BALB/c) background. In a given experiment the background, age and sex were matched. Mice were kept in isolators and fed autoclaved food and water *ad libitum*.

† Median values of a range.

‡ Mann–Whitney U test; $P < 0.05$ was considered to be significant.

§ NS, not significant.

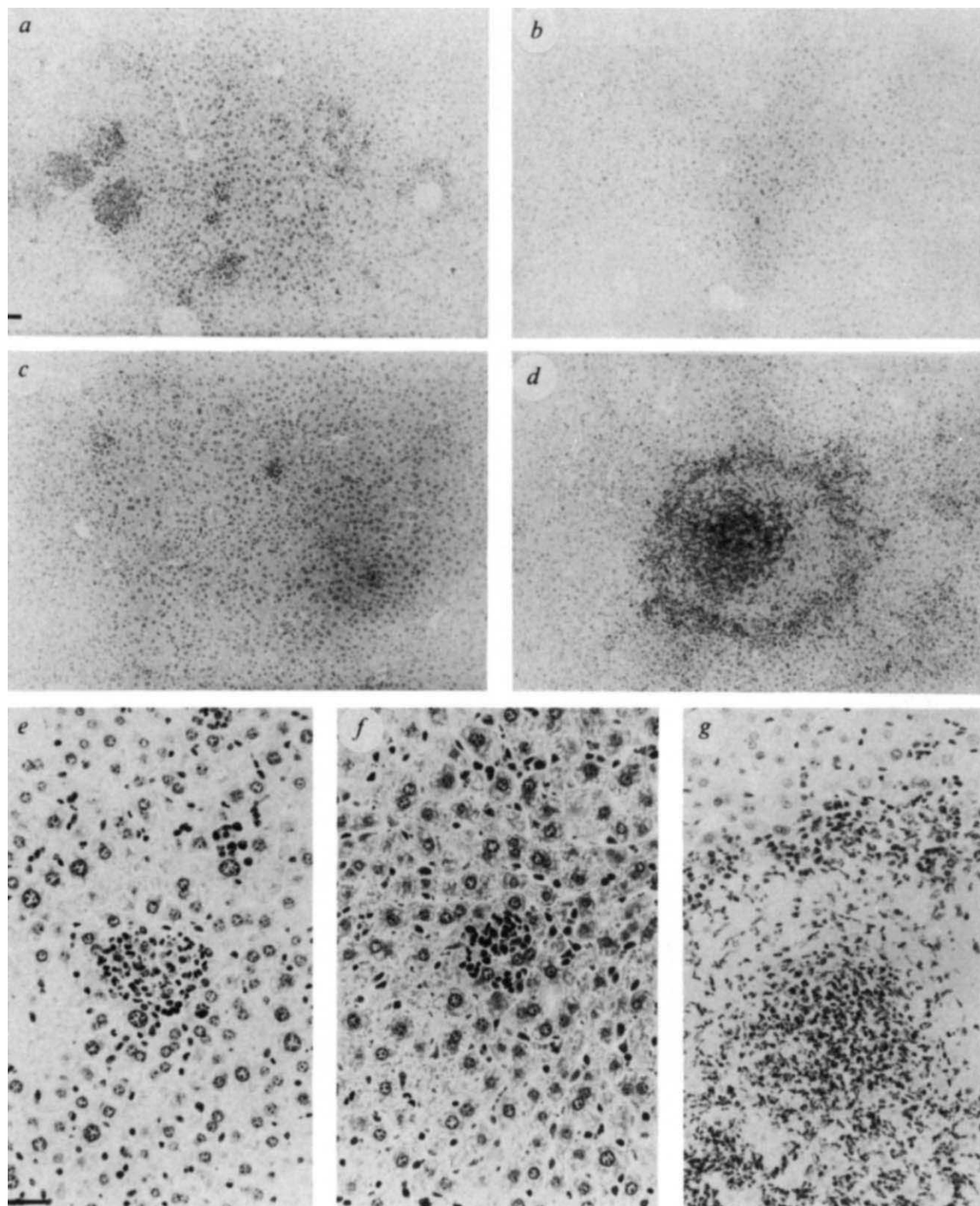


FIG. 1 Liver lesions in *L. monocytogenes*-infected TCR- β , TCR- δ , TCR- $\beta \times \delta$ mutants and control littermates. Mice were infected with *L. monocytogenes* ($\sim 5 \times 10^3$ CFU) and 7 d later their livers were removed. Sections representative of 10 to 15 animals per group are shown. a-d, Low magnification (100 $\times$; bar, 50 μ m); e-g, detailed view (400 $\times$; bar, 30 μ m). a and e, Liver section of control mice showing early granuloma formation. Granulomatous lesions (5 to 7 lesions per 100 $\times$ fields, 30-35 lesions per section) contained macrophages associated with a few granulocytes at the centre which were surrounded by lymphocytes. In peripheral regions, double-nucleated as well as macro-nucleated hepatocytes were detectable. b, Lack of lesions in TCR- $\beta \times \delta$ mutants. Although there was some increase in macrophages, indicating general activation of the reticulo-endothelial

system, distinct lesions were not identified. c and f, Lack of well-structured lesions in TCR- β mutants. A few small infiltrative lesions (2-3 lesions per 100 $\times$ fields, that is, about 15-20 lesions per section) containing granulocytes and macrophages but lacking lymphocytes were present. d and g, Development of abscess-like lesions in TCR- δ mutants (1-2 lesions per section). Acute inflammatory processes that manifested as abscess formation and were characterized by a centre of pycnotic granulocytes surrounded by a necrotic circle dominated in lesions of all 10 mice. Demarcation to the liver parenchyma was formed by an outer circle of macrophages, granulocytes and lymphocytes. Liver tissue was fixed in formalin, embedded in paraffin, cut to 4- μ m-thick sections, and stained with haematoxylin and eosin. Liver sections were evaluated for lesions per five random 100 $\times$ fields.

TABLE 2 DTH responses to listerial antigens and IFN- γ production by spleen cells in TCR- β and TCR- δ mutant and control littermates

Mutant	DTH (mm ⁻¹)*	IFN- γ secretion (U) [†]		
		<i>In vitro</i> restimulation	Cell number per well	
TCR- β ^{-/-}	0	HKL	2.8	0.1
		Con A	21.5	0.2
		Nil	0.1	0.1
TCR- δ ^{-/-}	15	HKL	>100	>100
		Con A	>100	>100
		Nil	0.9	0.1
Control	13	HKL	>100	>100
		Con A	>100	>100
		Nil	3.3	1.0

* Mice were infected with *L. monocytogenes* and on day 8, DTH reactions were elicited by injection of 50 μ l soluble listerial antigen into the right-hand footpad. After 24 h, footpad swelling was measured with a dial gauge caliper. DTH reactions represent differences between the right (injected) and left (non-injected) hind footpad. In uninfected control mice, footpad swelling was <2 mm⁻¹, excluding any influence of non-specific inflammatory response on DTH reactions. Medians of 5 mice per group.

[†] At day 9 after infection, spleen cells from *L. monocytogenes*-infected mice were cultured together with antigen (10⁷ heat-killed listeriae (HKL) per well) or with mitogen (1 μ g concanavalin A (Con A) per well) in supplemented Iscove's medium in round-bottomed microtitre plates²³. After 48 h, supernatants were collected and IFN- γ and interleukin-4 production measured by enzyme-linked immunosorbent assay (ELISA) as described²⁴. Interleukin-4 was virtually undetectable. Means are shown of at least three samples per group; s.d. <15%.

days (Table 1). All three types of mutant mice (TCR- α , TCR- β and TCR- δ mutants) were able to control early listeriosis (Table 1, experiments 1 to 7) as efficiently as heterozygous littermates used as controls. (Hereafter the latter mice are termed 'normal' littermates.) Treatment with anti-TCR- δ monoclonal antibody GL3 (ref. 13) exacerbated primary listeriosis in TCR- α and TCR- β mutants (Table 1, experiments 1 and 2), suggesting that

$\gamma\delta$ T cells are involved in clearance of infection in the $\alpha\beta$ T-cell-deficient mice. Mice carrying the mutations for both TCR- β and TCR- δ lacking all T lymphocytes^{11,12}, or mice homozygous for a mutation in the recombination activating gene-1 (RAG-1) and lacking T and B lymphocytes altogether¹⁴, were more sensitive to listeriosis than TCR- β mutants (Table 1, experiments 6 and 7). TCR- β $\times$ δ and RAG-1 mutants were incapable of clearing infection by three weeks. In contrast, at least some TCR- β mutants could achieve sterile clearance of listeriosis (Table 1, experiments 8 and 9). In some other TCR- β mutants, the bacterial counts at day 21 were ~1,000-fold lower than those in the T-cell-deficient animals (Table 1, experiment 9). These findings confirm the protective role of $\gamma\delta$ T lymphocytes in the absence of $\alpha\beta$ T cells.

Transient exacerbation of listeriosis by anti-TCR- δ or anti-CD4 plus anti-CD8 monoclonal antibody treatment has been reported^{4,15,17}. Earlier studies with mice carrying the genes for severe combined immunodeficiency (*SCID*) and *nude* provided evidence for T-cell-independent macrophage activation by natural killer cells as a major effector mechanism of early antilisterial immunity^{7,18}. But both of these types of T-cell-deficient mice become chronic carriers of *L. monocytogenes* and succumb to the disease after several weeks.

Delayed-type hypersensitivity (DTH) is considered to reflect the part of protective immunity conferred by CD4⁺ T cells against intracellular bacteria^{1,2,8,19}. DTH responses against listerial antigens were equally strong in TCR- δ mutant and normal littermates, but were absent in TCR- β mutants, suggesting that DTH is exclusively dependent on $\alpha\beta$ T cells and independent of $\gamma\delta$ T cells (Table 2). Macrophage activation by interferon (IFN)- γ is thought to be central to protection against intracellular bacteria^{2,20,21}. We observed increased IFN- γ production by spleen cells from TCR- δ mutants and controls after antigen and mitogen stimulation (Table 2). We assume that concanavalin A (con A) causes IFN- γ secretion in both natural killer cells and T cells, reflecting maximal IFN- γ production, whereas IFN- γ production by antigen (heat-killed listeriae) is restricted to T cells. In TCR- β mutants only marginal amounts of IFN- γ were observed after antigen stimulation. These findings show that $\alpha\beta$ T cells are the principal source of antigen-induced IFN- γ pro-

TABLE 3 Secondary listeriosis in TCR- β , TCR- α , TCR- δ and TCR- β $\times$ δ mutant mice*

Experiment (group)	Type of mice	No. of mice	Vaccination		Secondary infection (day 0)			
			Day of inoculation	Dose (in log ₁₀)	Dose (in log ₁₀)	Day of testing	CFU per spleen (in log ₁₀) [†]	Significant difference in CFU [‡]
1(1)	TCR-β ^{-/-}	5	-14	3.3	4.5	3	3.7 (2.9-3.9)	vs 1(2)
1(2)	Control	5	-14	3.3	4.5	3	<2 (<2-<2)	vs 1(1)
1(3)	TCR-β ^{-/-}	5	-14	3.3	4.5	6	2 (<2-3.5)	NS§
1(4)	Control	5	-14	3.3	4.5	6	<2 (<2-<2)	NS
2(1)	TCR-α ^{-/-}	5	-14	3.2	4.4	3	3.8 (3.6-6.1)	vs 2(2)
2(2)	Control	5	-14	3.2	4.4	3	2.8 (<2-3.7)	vs 2(1)
2(3)	TCR-α ^{-/-}	5	-14	3.2	4.4	6	3.0 (<2-5.7)	NS
2(4)	Control	5	-14	3.2	4.4	6	<2 (<2-3.9)	NS
3(1)	TCR-α ^{-/-}	5	-17	3.8	5.1	2	4.6 (3.9-5.0)	vs 3(2), vs 3(3),
3(2)	TCR-α ^{-/-} + anti-TCR-δ	6	-17	3.8	5.1	2	5.5 (4.7-5.8)	vs 3(1), vs 3(3)
3(3)	Control	5	-17	3.8	5.1	2	<2 (<2-4.3)	vs 3(1), vs 3(2)
4(1)	TCR-δ ^{-/-}	5	-15	3.3	5.3	3	2.0 (<2-2.2)	NS
4(2)	Control	5	-15	3.3	5.3	3	2.0 (<2-2.0)	NS
4(3)	TCR-δ ^{-/-}	5	-15	3.3	5.3	8	<2 (<2-<2)	NS
4(4)	Control	5	-15	3.3	5.3	8	<2 (<2-<2)	NS
5(1)	TCR-β ^{-/-}	5	-15	3.0	4.8	3	3.7 (3.6-3.9)	vs 5(2), vs 5(3), vs 5(4)
5(2)	TCR-β × δ ^{-/-}	5	-15	3.0	4.8	3	5.1 (4.2-5.4)	vs 5(1), vs 5(4)
5(3)	RAG-1 ^{-/-}	4	-15	3.0	4.8	3	5.4 (4.3-5.5)	vs 5(1), vs 5(4)
5(4)	Control	5	-15	3.3	4.8	3	<2 (<2-<2)	vs 5(1), vs 5(2), vs 5(3)

* Mice were vaccinated with the indicated sublethal dose of *L. monocytogenes* 14 to 17 d before secondary infection (day 0). Between 5-7 d before the secondary infection, mice were treated with ampicillin (twice, subcutaneously, with 10 mg each time; ref. 23). On the indicated day after secondary infection, CFU in spleens were determined. All mice used were of (129 $\times$ C57BL/6) or (129 $\times$ BALB/c) background and were more than 10 weeks old. In a given experiment, the genetic background, age, and sex were matched. Mice were kept in isolators and fed autoclaved food and water *ad libitum*.

[†] Median values of a range.

[‡] Mann-Whitney *U* test; *P* < 0.05 was considered to be significant.

[§] NS, not significant.

duction in listeriosis, with $\gamma\delta$ T cells making only a minor contribution. This may mean that antilisterial resistance mediated by $\gamma\delta$ T cells involves mechanisms other than IFN- γ secretion²².

TCR mutants were next vaccinated with viable bacteria, treated with ampicillin to ensure complete eradication of any remaining bacteria²³, and subsequently challenged with a lethal dose of bacteria (Table 3). Control experiments had shown that all groups of mice died by day 2 at inocula of $\geq 5 \times 10^4$ (log 4.6) *L. monocytogenes* organisms unless they had been vaccinated (data not shown). As expected, normal littermates were fully protected by vaccination (Table 3, experiments 1 to 5). Vaccinated TCR- δ mutants were equally resistant (Table 3, experiment 4). In contrast, secondary listeriosis was markedly exacerbated in vaccinated TCR- α and TCR- β mutants (Table 3, experiments 1, 2, 3 and 5) compared with the vaccinated normal littermates or vaccinated TCR- δ mutants. Secondary listeriosis was even more pronounced, however, in TCR- $\beta \times \delta$ double mutants, RAG-1 mutants (Table 3, experiment 5) or in TCR- α mutants treated with the anti-TCR- δ antibody (Table 3, experiment 3), indicating that $\gamma\delta$ T cells do have a role in vaccine-induced immunity in the $\alpha\beta$ T-cell-deficient mice. The failure of $\gamma\delta$ T cells to compensate fully for $\alpha\beta$ T cells in this immunity could be quantitative or qualitative in nature. In the $\alpha\beta$ T-cell-deficient mice and normal littermates, few $\gamma\delta$ T cells are present (1–10%) compared with the number of $\alpha\beta$ T cells in normal littermates. The absolute numbers of T cells must influence the kinetics of infectious diseases, or perhaps the $\gamma\delta$ T cells did not develop their full protective potential after *L. monocytogenes* vaccination. The $\gamma\delta$ T cells isolated from mice after secondary infection were potent IFN- γ producers after antigen stimulation *in vitro* (C. Ladel and S.H.E.K., unpublished), indicating that further characterization of this cytokine will be necessary to determine its *in vivo* role in TCR- β mutants using either *in vivo* reconstitution with recombinant IFN- γ or *in vivo* neutralization with anti-IFN- γ antibody^{20,21}.

Lesions in infected normal littermates were granulomatous, although epithelioid cells did not develop (Fig. 1a and e). In contrast, in infected anti-TCR- δ -antibody-treated, TCR- β mutants (data not shown) and in infected TCR- $\beta \times \delta$ double mutants (Fig. 1b), liver lesions were virtually absent. Livers of infected TCR- β mutant mice not only contained two- to three-fold fewer lesions, but these were also smaller than and unlike those found in infected normal littermates: they showed phagocyte infiltration only and lacked any organoid structure (Fig. 1c and f). We also noted the appearance of large atypical lesions in livers of $\gamma\delta$ T-cell-deficient mice that had been infected with *L. monocytogenes* (Fig. 1d and g). The pronounced inflammation was characterized by pronounced abscess formation, not reported previously for infections of normal mice with intracellular bacteria. These histological findings indicate that in primary listeriosis: (1) $\alpha\beta$ T cells are crucial for the development of granulomatous lesions; (2) $\gamma\delta$ T cells are not sufficient for the generation of such organoid lesions, but that (3) $\gamma\delta$ T cells regulate $\alpha\beta$ T-cell functions so that in their absence $\alpha\beta$ T cells induce numerous abscess-like lesions rather than granulomatous lesions.

In summary, our studies of primary *L. monocytogenes* infections of mice selectively deficient in $\alpha\beta$ or $\gamma\delta$, or both types of T cells, have demonstrated that either T-cell subset can confer protective immunity against early listeriosis in the absence of the other subset. In the secondary infection, the protective role of $\gamma\delta$ T cells is limited but still significant. In addition, our results indicate that $\gamma\delta$ T cells may regulate (suppress) the formation of liver lesions in listeriosis. We aim to investigate the mechanisms of this regulatory function and to determine whether this finding is generalizable to other types of immune response. □

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Spondylometaphyseal dysplasia in mice carrying a dominant negative mutation in a matrix protein specific for cartilage-to-bone transition

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THE vertebrate skeleton is formed primarily by endochondral ossification, starting during embryogenesis when cartilage anlagen develop central regions of hypertrophic cartilage which are replaced by bony trabeculae and bone marrow^{1,2}. During this process chondrocytes express a unique matrix molecule, type X collagen³. We report here that mice carrying a mutated collagen X transgene develop skeletal deformities including compression of hypertrophic growth plate cartilage and a decrease in newly formed bone, as well as leukocyte deficiency in bone marrow, reduction in size of thymus and spleen, and lymphopenia. The defects indicate that collagen X is required for normal skeletal morphogenesis and suggest that mutations in COL10A1 are responsible for certain human chondrodysplasias, such as spondylometaphyseal dysplasias and metaphyseal chondrodysplasias⁴.

The programmed tissue substitution of endochondral ossification (EO) leads to the emergence of growth plates that provide bones with longitudinal growth potential. Within growth plates a chondrocytic differentiation gradient culminates in a zone of hypertrophy where collagen X is a unique and major product^{3,5,6}. To define its regulation and function in EO, we generated transgenic mice with a dominant negative mutation in collagen X. Transgene constructs were designed assuming that homotrimeric collagen X molecules, composed of $\alpha 1(X)$ subunits, assemble through interactions of carboxy-terminal domains followed by folding of the triple-helix from the carboxy to the amino end⁷ (Fig. 1c). The constructs contained either

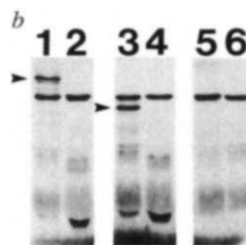
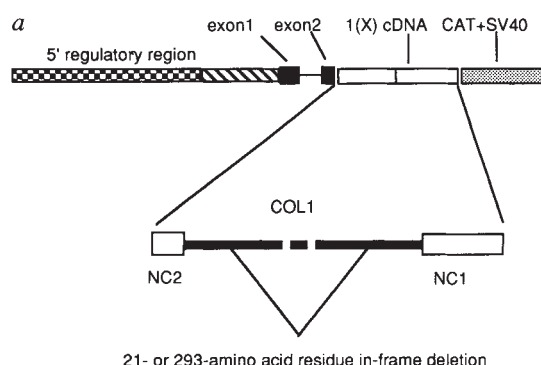
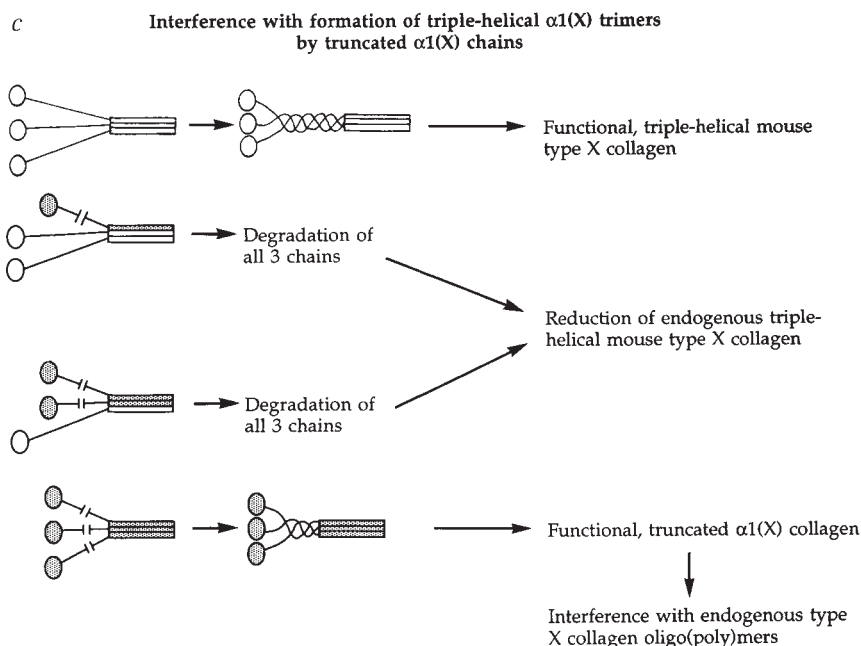


FIG. 1 Design of chicken $\alpha 1(X)$ DNA constructs. **a**, Four constructs were prepared by ligating a chicken $\alpha 1(X)$ cDNA containing a central in-frame deletion of either 21 or 293 codons to the 5' portion of exon 2 in the chicken $\alpha 1(X)$ gene. The 5' region of the gene contains repressor elements located within 4,700 bp (chequered and cross-hatched bars) or within 1,600 bp (cross-hatched bar) 5' of the transcription start site¹⁰. Downstream of the cDNA, the bacterial chloramphenicol acetyl transferase (CAT) gene with additional splice signals and SV40 polyadenylation site was added. Expression of constructs yields truncated $\alpha 1(X)$ polypeptides with 21 or 293 amino-acid deletions within the triple-helical domain (COL 1), but with intact NC1 and NC2 non-triple-helical domains. **b**, [³⁵S] Methionine-labelled cell-free translation products synthesized from RNA made *in vitro* from the truncated chicken $\alpha 1(X)$ cDNAs. Lanes 1, 2, 0.25 μ g template RNA with 21 codon deletion; lanes 3, 4, 0.25 μ g template RNA with 293 codon deletion; lanes 5, 6 are translation controls; lanes 2, 4, 6 contain products digested with bacterial collagenase. Arrows indicate collagenase-sensitive translation products, migrating below the 69K globular standard in lane 4 (~58K collagenous band), and below the 43K globular standard in lane 4 (~39K collagenous band), as predicted. **c**, Schematic representation of hypothetical mechanisms through which truncated chicken $\alpha 1(X)$ polypeptides (shaded molecules) may interfere with formation of triple-helical mouse $\alpha 1(X)$ homotrimers (non-shaded molecules), or with their oligo (poly)mer structure in the matrix.

METHODS. The 21-codon deletion within the $\alpha 1(X)$ cDNA was generated by removal of a 63-bp *Apal* fragment (nucleotides 1,013–1,075¹⁷) in the cDNA SPLX⁹, followed by religation. To generate the 293-codon deletion, 905-bp *Apal*–*Apal*–*Apal* fragments were removed from SPLX⁹ (nucleotides 170–1,085)¹⁷, and the adaptor 5'-ACC TGG TCC GGC CGG ACC AGG TGG CC-3' was inserted. In-frame deletions were confirmed by sequencing. Cloning of cDNAs into the Sp65 vector enabled *in vitro* transcription of the cDNAs, and subsequent cell-free translation confirmed generation of truncated polypeptides. To generate the constructs 1600-21 and 1600-293, a 2.3-kilobase (kb) *NsiI*–*NsiI* fragment was isolated from the chicken genomic clone PL10⁹ (including 1,600 bp of sequence upstream of the transcription start, exon 1, intron 1 and exon 2 up to the translation start codon). The fragment was blunt-ended with the exonuclease activity of T4 polymerase to remove the translation start codon, *Sall* linkers were added, and the resulting fragment was



ligated into the *Sall* site of the polylinker region of pBLCAT3¹⁸. The two cDNA fragments containing in-frame deletions of either 21 or 293 codons were isolated from the Sp65 vector with *EcoRI* and *HindIII*, and blunt-ended. After addition of *XbaI* linkers the fragments were inserted into the *XbaI* site of the polylinker region of pBLCAT3 containing the 2.3-kb $\alpha 1(X)$ gene fragment. To generate the constructs 4700-21 and 4700-293, a 1.1-kb *HindIII*–*HindIII* fragment (corresponding to the 5' portion of the chicken $\alpha 1(X)$ regulatory region) was removed from the 1600-21 and 1600-293 constructs. A 4.2-kb *EcoRI*–*HindIII* fragment consisting of more 5' region of upstream regulatory elements was isolated from PL10⁹, blunt-ended, and after addition of *HindIII* linkers, was ligated into this *HindIII* site. The recombinant DNAs were purified twice on a CsCl gradient, and the inserts were isolated from vectors with *NotI* and *KpnI*. Following purification by agarose gel electrophoresis, the insert DNAs were recovered by electroelution, concentrated/desalted by ion exchange chromatography (Schleicher and Shuell Elutip), and resuspended in 10 mM Tris, 0.25 mM EDTA pH 7.5 at 25–390 ng μ l⁻¹ following ethanol precipitation. Microinjection into fertilized eggs was done by DNX, Inc. with the support of a contract from the National Institute of Child Health and Human Development.

4,700 base pairs (bp) or 1,600 bp of the 5' region of the chicken $\alpha 1(X)$ gene, and a chicken $\alpha 1(X)$ complementary DNA with either a small (21 codons) or a large (293 codons) in-frame deletion in the triple-helical coding domain (Fig. 1a). Given the high degree of homology between chicken and mouse $\alpha 1(X)$ carboxyl domains⁸, the truncated chicken polypeptides should compete with endogenous mouse chains for assembly, and thus

interfere with proper triple-helical folding of trimers (Fig. 1c). The 5' region of the chicken $\alpha 1(X)$ gene, used to drive expression of the constructs (Fig. 1a)⁹, contains promoter and silencer elements active in both embryonic chicken (fibroblasts, small chondrocytes, hypertrophic chondrocytes)¹⁰, and in mammalian (RCJ¹¹; data not shown) cells. By combining either the long or the short 5' upstream region with the small or the large in-frame

FIG. 2 Detection of transgene and its product. **a**, Southern blots of DNA from hemizygous mice derived from 10 independent transgenic lines, representing all 4 constructs. Mouse genomic DNA was isolated from tails¹⁹, 8 µg were digested for 6 h with *SacI*, electrophoresed in a 0.8% agarose gel, blotted onto nylon, and hybridized with a ³²P-labelled chicken $\alpha 1(X)$ cDNA probe at 45 °C with formamide. The restriction map of the constructs (compare with Fig. 1), the expected fragments after *SacI* digestion, and the 2.4-kb $\alpha 1(X)$ chicken probe are indicated in the diagram on the left. The 10 transgenic lines have yielded similar phenotypes, and independent integration sites of the transgene are observed between lines. In mice carrying constructs with a 21-codon deletion, major bands of 5.4 kb and 0.6 kb are seen. The 5.4-kb band corresponds to the combination of 1.8-kb and 3.6-kb bands due to concatenation of several copies of the construct at the integration site; the 0.6-kb band corresponds to the internal 0.6-kb fragment covered by the probe. Likewise, in mice carrying constructs with the 293-codon deletion, the 5.2-kb band corresponds to a combination of 3.4-kb and 1.8-kb bands due to concatenation of the construct DNA at the integration site. In lines with this large deletion, the 0.6-kb band is not seen because the deletion eliminates the *SacI* site at the 3' end of the 0.6-kb fragment (see diagram at left). Additional bands seen in each line correspond to the 3' insertion sites, and transgene arrangements. A single integration site is seen in lines 5-1, 4-2, 3-2, and 3-4, with 5-1, 4-2, and 3-2 having combinations of 3'-to-5' and 5'-to-3' transgene arrangements. Double integration sites are probably present in lines 5-4, 1-5, 3-1, and perhaps 4-1. Line 1-3 contains a single copy of the transgene, therefore, a ~3.6-kb band corresponding to the 3' portion of the transgene and its flanking sequence is present, rather than the internal 5.4-kb band (due to concatenation of several construct copies). In lines 1-3 and 1-5, the 0.6-kb bands are not visible because of the low copy numbers. **b-e**, Immunohistochemical localization of chick collagen X in 1-day-old F₁ littermates from transgenic line 3-2. The metaphyseal regions of tibia are shown from genotypically negative (**b**), and genotypically positive (**c**, **d**) littermates, reacted with anti-chicken type X collagen monoclonal antibodies¹² 6F6 (**b**, **c**, **e**), and 1A6 (**d**). **e**, Day-18 chick embryonic sterna showing transition zone from hypertrophic collagen X-producing chondrocytes to type X negative cells. Note localization of the transgene protein to the upper hypertrophic cartilage zones of tibia from genotypically positive pups.

METHODS. Cryosections (6 µm) of OCT-embedded tissue were fixed in acetone-methanol (1:1, vol:vol) (1 min), rinsed in PBS (15 min, 3 changes), treated with 1 mg ml⁻¹ testicular hyaluronidase (30 min, 37 °C), and again washed in PBS (15 min, 3 changes). The sections were treated with 5% H₂O₂ in methanol (15 min), rinsed in 4% fetal calf serum in PBS (15 min, 3 changes), and incubated with the primary antibody¹² (6F6 or 1A6 ascites fluid diluted 1:100) (room temperature,

2 h). After rinses with 4% FCS in PBS (15 min, 3 changes), the sections were treated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (diluted 1:1,000) (room temperature, 30 min) and rinsed again in 4% FCS in PBS. Slides were conditioned in 0.1 M Tris-HCl buffer pH 7.5 (30 min), and peroxidase activity was visualized by reacting the sections with a diaminobenzidine solution containing hydrogen peroxidase. Slides were then dehydrated, cleared and mounted with Permount.

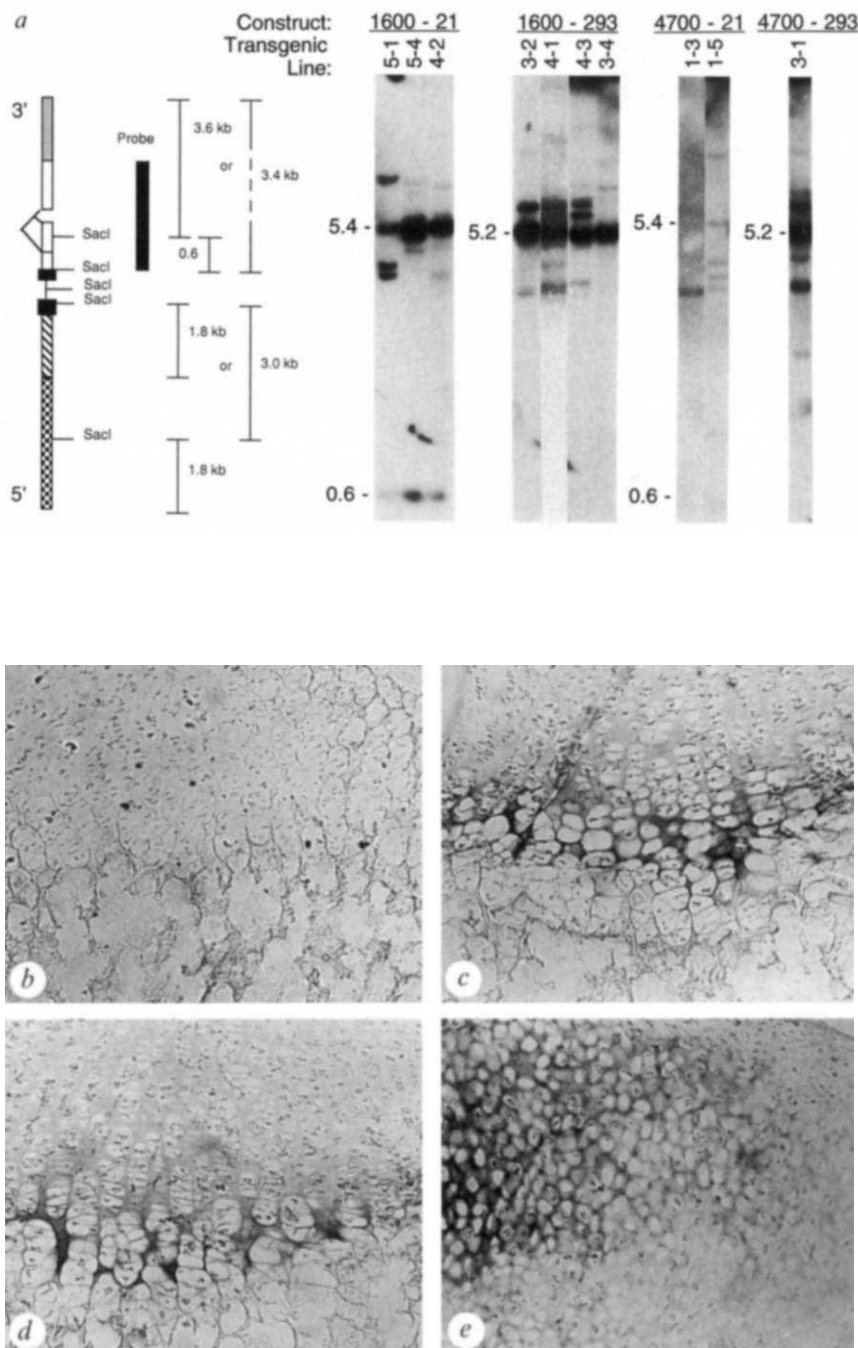
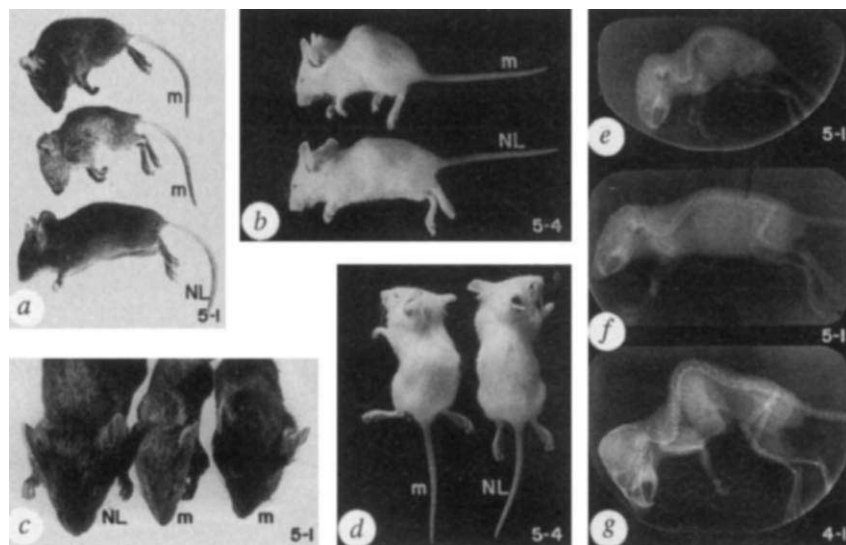


FIG. 3 Comparison of hemizygous mutant (M) and normal (NL) littermates from independent transgenic mouse lines 5-1 and 5-4. At ~day 17 after birth, affected pups acquire a hunched back (a, b, d), paresis of hind limbs (d), inset eyes and narrow snouts (c), and begin to waste. X-ray analysis reveals an accentuated neck lordosis and a thoracolumbar kyphosis in the mutant (e), but not in the genotypically negative littermate (f). g, X-ray of a founder mouse from transgenic line 4-1 displaying a similar phenotype at 6 months of age.



deletion within the coding region, four collagen X constructs were generated: 4700–21, 4700–293, 1600–21 and 1600–293.

The constructs were microinjected (DNX, NJ) into pronuclei of mouse eggs from C57BL/6 × SJL F₂ hybrid mice. Southern blot analysis identified 21 founders from a total of 68 mice. Breeding of founders with C57BL/6 × D₂F₁ (B₆D₂F₁) mice established 19 transgenic lines, each with an independent transgene integration site (Fig. 2a). So far, 14 lines representing all constructs have yielded mice with similar skeletal and growth abnormalities, indicating that the phenotype is not caused by disruption of an endogenous gene through transgene insertion.

Transgene expression was confirmed by northern blots (not shown), and by localization of the transgene protein in 1-day-old homozygotes from lines 1-3 (4700–21) and 3-2 (1600–293) (Figs 2b–e). Chick collagen X was localized to hypertrophic cartilage matrices of long bones by two independently derived monoclonal antibodies against chick collagen X¹² (Fig. 2c, d); this suggested that hypertrophic chondrocytes are actively secreting proteins, making it unlikely that transgene expression has a nonspecific ‘poisoning’ effect on the cells. Staining was also observed in the 1–2 cell layers of hypertrophic cartilage in vertebrae and in intervertebral discs¹³, whereas brain, heart, thymus, lung, liver, kidney and spleen from line 3-2 (1600–283) 1-day-old pups were negative (not shown). Therefore, within the sensitivity of immunohistochemical detection, tissue-specific expression of transgene protein indicates that the chicken $\alpha 1(X)$ promoter and 5' elements are functional in mice.

Most transgenic pups were visually indistinguishable from control littermates until postnatal day 16/17, when 15–20% of the genotypically positive pups developed progressive hunching of the back, gradual hind limb paresis, respiratory problems, and died within 4 days (Fig. 3). Lordosis of the cervical vertebral column and thoracolumbar kyphosis was seen only in animals carrying a double dosage of the transgene in certain lines, but in most lines in hemizygotes as well. Homozygous litters from 8 lines also yielded pups with significant dwarfism which persisted into adulthood. Many founders were mosaic with no noticeable abnormalities; however, a few had defects such as a short stature and trunk, cranial bulge, abnormal gait, progressive thoracolumbar kyphosis, collapsing pelvis, and bowed legs. With age, several founders developed secondary infections and tumours, suggesting an impaired immune system (see below).

Histology of over 30 transgenic mice revealed similar abnormalities in growth plates, bony trabeculae, and bone marrow of

all tissues undergoing EO, although the severity of the phenotype varied among different lines. First, resting and proliferating chondrocytes appeared unaffected in most lines, while the extent of hypertrophy was reduced. Hypertrophic chondrocytes also had pycnotic nuclei and were flattened, as if the matrix around them had collapsed (Fig. 4). More severe phenotypes included compression within all regions of growth plates, disorganization of chondrocyte columns, and absence of mature hypertrophic cells (not shown). Second, the number and size of bony trabeculae, composed of calcified hypertrophic cartilage cores with newly deposited bone, was reduced (Fig. 4). Despite this reduction, no mineralization abnormalities in the trabeculae and periosteal bone were detected by Alizarin red S staining (not shown). Third, the bone marrow showed a predominance of erythrocytes, and a paucity of leukocytes in the 15–20% of the genotypically positive hunch-backed mice (Fig. 4). Peripheral blood analysis showed a normal haematocrit, but a reduction in leukocytes. Affected lymphatic organs included a dramatically reduced thymus with a paucity of cortical lymphocytes, and a small and occasionally discoloured spleen.

Localization of the transgene product (Fig. 2c,d) in areas showing defects (Fig. 4) suggests that the phenotype results from disruption of mouse collagen X function. Growth plate compression supports the hypothesis that collagen X provides the structural support in hypertrophic cartilage matrices undergoing degradation. Collagen X may fulfil this function by forming pericellular polymeric networks seen *in vitro*¹⁴, similar to those formed by the homologue collagen VIII, in the Descemet's membrane^{3,15}. The mouse abnormalities therefore may result from collapse of a collagen X-containing pericellular lattice due to a reduced level of intact collagen X molecules. Onset of the hunch-back phenotype correlates with weaning and increase in the pups' mobility. Perhaps increased mechanical stress on an already weakened skeleton causes growth plate compression and local deformation of vertebrae. The correlation with lymphopenia is intriguing; perhaps alterations in hypertrophic cartilage and bone microenvironments inhibit proper haematopoietic cell differentiation.

The skeletal and growth abnormalities in the transgenic mice resemble certain human spondylometaphyseal dysplasias (SMD) and metaphyseal chondrodysplasias (MCD)⁴. Recent studies have shown that affected members of a kindred with the autosomal dominant disorder Schmid MCD contain a 13-bp deletion in one collagen X allele¹⁶. The likely consequence of the mutation

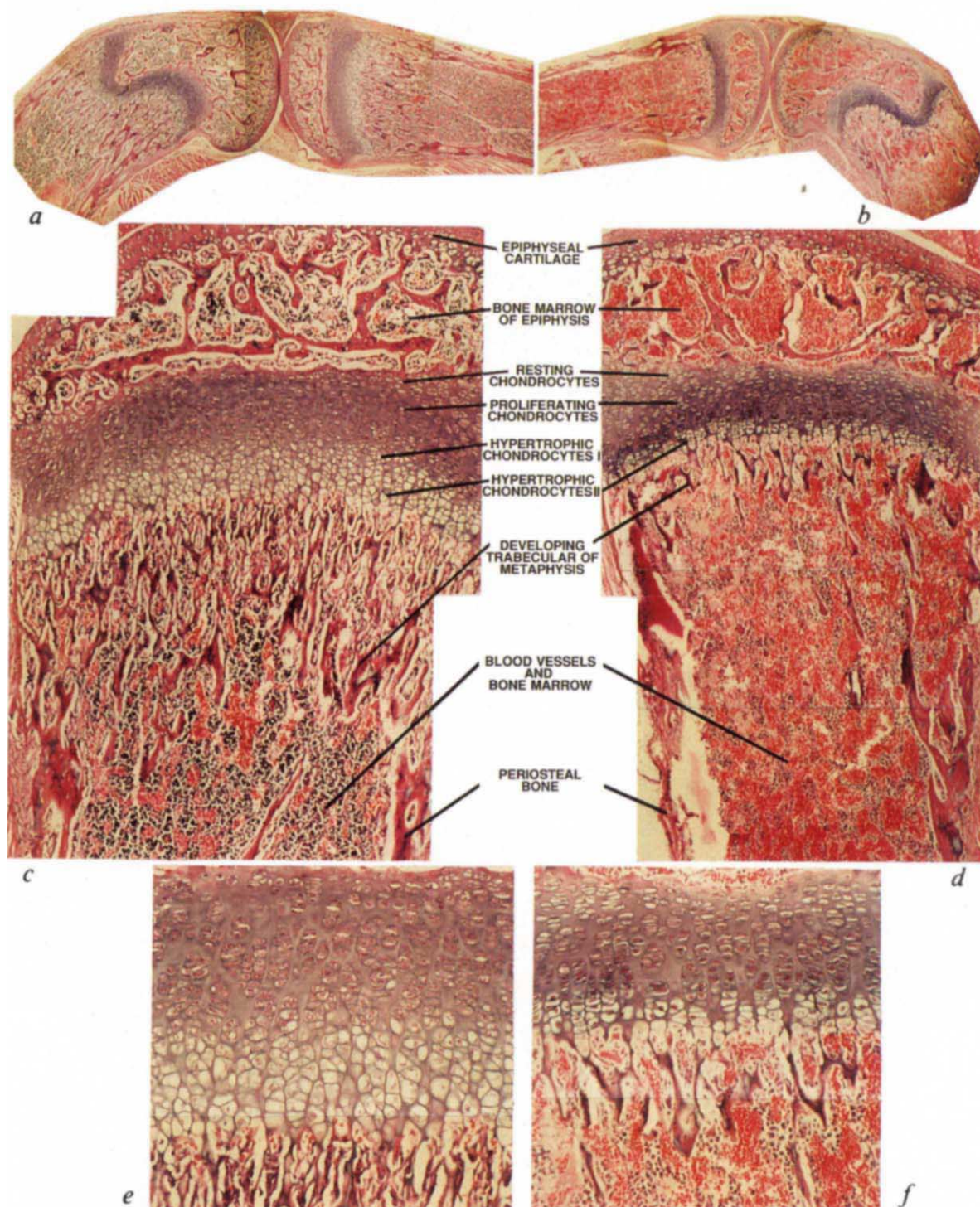


FIG. 4 Haematoxylin and eosin staining of decalcified 5- μ m longitudinal sections of hind limbs from genotypically negative normal (a, c, e) and genotypically positive hemizygous mutant (b, d, f) littermates at day 21 after birth, from transgenic line 5-1. The tissues were fixed in Hollande Bouin's solution for 3 days and decalcified overnight in 4% formaldehyde, 1% sodium acetate, 10% disodium EDTA. a, b, Low-magnification montage showing the metaphyseal region of the femur and tibia. c, d, Higher magnification of tibia shown in a, b. The following alterations are seen in the mutant: (1) compressed, more basophilic matrix in the zone of chondrocyte hypertrophy; (2) lack of zone 1 hypertrophic chondrocytes; (3) reduced degree of chondrocyte hypertrophy in

zone 2; (4) pycnotic nuclei in hypertrophic chondrocytes; (5) decrease in number and length of calcified trabeculae both in the secondary centre of ossification and in the metaphysis; and (6) a predominance of erythrocytes in the bone marrow, with a reduction in leukocytes. Epiphyseal cartilage and chondrocytes in the resting and proliferating zones, as well as calcification of trabeculae, and periosteal bone formation, do not appear to be affected in the hemizygous mutant. e, f, Enlargement of the tibial growth plates shown in c, d. Note the reduction in thickness of the mutant growth plate (f), and the altered morphology of the hypertrophic chondrocytes as described above.

is inability of the mutant chain to participate in trimer assembly, leading to a 50% reduction in collagen X. We suggest that the mechanism of growth plate abnormalities in Schmid MCD is similar to that in the mice. The transgenic mice described here will, therefore, not only provide insights into collagen X regulation and function during EO, but can also serve as models for chondrodysplasias with spondylometaphyseal involvement. □

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Molecular characterization of a peripheral receptor for cannabinoids

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THE major active ingredient of marijuana, Δ^9 -tetrahydrocannabinol (Δ^9 -THC), has been used as a psychoactive agent for thousands of years. Marijuana, and Δ^9 -THC, also exert a wide range of other effects including analgesia, anti-inflammation, immunosuppression, anticonvulsion, alleviation of intraocular pressure in glaucoma, and attenuation of vomiting¹. The clinical application of cannabinoids has, however, been limited by their psychoactive effects, and this has led to interest in the biochemical bases of their action. Progress stemmed initially from the synthesis of potent derivatives of Δ^9 -THC^{4,5}, and more recently from the cloning of a gene encoding a G-protein-coupled receptor for cannabinoids⁶. This receptor is expressed in the brain but not in the periphery, except for a low level in testes. It has been proposed that the non-psychoactive effects of cannabinoids are either mediated centrally or through direct interaction with other, non-receptor proteins^{1,7,8}. Here we report the cloning of a receptor for cannabinoids that is not expressed in the brain but rather in macrophages in the marginal zone of spleen.

To identify novel G-protein-coupled receptors expressed in myeloid cells, polymerase chain reaction (PCR) using degenerate primers was done on complementary DNA prepared from the human promyelocytic leukaemic line HL60. Treatment of HL60

cells with dimethylformamide (DMF) induces granulocyte differentiation, whereas tetradecanoylphorbol acetate (TPA) induces macrophage differentiation⁹. Amplification products from DMF-treated cells were cloned and sequenced, and six classes of clone showed homology to the G-protein-coupled receptor family. Two of these classes corresponded to previously identified receptors; the interleukin-8 receptor-B (ref. 10) and the adenosine A3 receptor¹¹ (S.M., manuscript in preparation). Of the remaining four sequences, only one showed particular homology to a published receptor. This clone, CX5, was related to a cannabinoid receptor cloned originally from rat brain⁶. To investigate the functional significance of this homology, the CX5 insert was used to screen an HL60 cDNA library. Two cDNA clones were obtained, hCX5.1 and hCX5.36, the latter extending the furthest 5' and the complete nucleotide sequence of this clone is shown in Fig. 1a. The protein encoded by hCX5.36 shows 44% identity with the human cannabinoid receptor and the degree of identity rises to 68% for those transmembrane residues proposed to confer ligand specificity¹².

To determine if the CX5 receptor binds cannabinoids, the hCX5.36 cDNA was inserted into an expression vector and transfected into tissue culture cells. Figure 2a shows a binding curve of the cannabinoid receptor ligand Win 55212-2 (ref. 13) to membranes prepared from the transfected cells. The control cells do not express receptors for cannabinoids, but expression of hCX5.36 causes the appearance of a saturable number of high-affinity binding sites for WIN 55212-2 and also for a second high-affinity cannabinoid CP55,940 (ref. 5; Fig. 2b, and data not shown). The affinities of the receptor for these structurally unrelated ligands (Win 55212-2: dissociation constant K_d 3.7 nM (+/-0.4 nM); CP55,940: K_d 1.6 nM (+/-0.5 nM)), are comparable to the analogous figures (24 nM and 2–15 nM) reported for the brain receptor^{13–15}. Furthermore, competition binding analysis showed that the CX5 receptor can distinguish between closely related derivatives of the archetypal cannabinoid Δ^9 -THC, showing a lower affinity for the relatively inactive cannabidiol, than for the biologically active Δ^9 -THC, cannabinol and 11-OH- Δ^9 -THC (Fig. 2b). Thus it appears that hCX5.36 encodes a selective, high-affinity receptor for cannabinoids. Note that although cannabinol is only weakly cannabimimetic and binds the brain receptor with an affinity about 10-fold less than that of Δ^9 -THC^{5,14}, this ligand binds to the CX5 receptor with an affinity comparable to that of Δ^9 -THC (250 nM versus 320 nM, Fig. 2b). This suggests that cannabinol may have a preference for the CX5 receptor over the brain receptor. Recently, a novel compound isolated from brain, arachidonyl ethanolamide (anandamide), has been identified as a candidate ligand for the brain receptor¹⁶ and Fig. 2b shows that this compound can also bind to the CX5 receptor. The binding affinity (K_i 1.6 μ M (+/-0.4)) is lower than that reported for brain membranes (K_i 52 nM), but it should be noted that the apparent receptor affinities of cannabinoids can vary depending on the assay system used^{5,14,15}. In the following text we shall refer to the original receptor as CB-R and this new receptor as CX5.

When CX5 was used to probe northern blots of RNA from HL60 cells it hybridized to two transcripts of about 2.5 and 5.0 kilobases (kb) (Fig. 3a). The two transcripts probably arise from the use of alternative poly(A)⁺ addition sites. Clone hCX5.1 does not have a poly(A)⁺ tail at the position of that in hCX5.36, but instead extends further in the 3' direction (Fig. 1a). The putative polyadenylation sequence of hCX5.36 (GAUAAA) is a variant of the AAUAAA consensus that is found in a small fraction of messages and which can be used, albeit inefficiently, *in vitro*¹⁷. CX5 is expressed in uninduced HL60 cells, but transcript levels are elevated further on myeloid, or granulocyte, differentiation, although the gene does not appear to be expressed in mature neutrophils isolated from blood (data not shown).

To investigate the tissue distribution of CX5, a portion of a rat homologue was isolated by PCR. This rat probe (rCX5) detects an mRNA of about 2.5 kb in spleen, but not in a variety

FIG. 1 Nucleotide and protein sequences of the cDNAs hCX5.36 and hCX5.1. *a*, Nucleotide sequence of the hCX5.36 cDNA and the protein sequence encoded by the longest open reading frame, the in-frame stop codon upstream of the most 5' ATG is underlined. Also shown is the nucleotide sequence of hCX5.1 where it diverges from that of hCX5.36 after the putative poly(A) addition site (underlined). *b*, Comparison of the protein encoded by hCX5.36 with the previously reported human cannabinoid receptor²⁸. Identities are boxed and the seven putative transmembrane segments are underlined. METHODS. Oligo-dT primed cDNA was synthesized from poly(A)⁺ RNA prepared from HL60 cells induced with 0.5% DMF for 3 days. cDNA (5 ng in 20 µl) was amplified with *Taq* polymerase using degenerate primers encoding regions conserved between many G-protein-coupled receptors. Those that produced CX5 were GAGGGCCATYISNNTNGAYMGNTA and TGAAGCTTSHRTANANSANNNGRTT (en-coded regions in bold in sequence alignment). 40 cycles of 94 °C, 1 min, 50 °C, 2 min and 72 °C 2 min, in 10 mM Tris-HCl pH 8.3, 3 mM MgCl₂, 100 mM tetramethylammonium chloride, 0.05% Tween 20, 0.05% NP-40, 250 µM dNTPs, 20 µM each primer. Gel-purified amplification products were digested with *Apal* and *HindIII* and cloned into Bluescript. After classification of the products by sequencing, the insert from clone CX5 was used to screen 2 × 10⁵ colonies of a cDNA library from TPA-treated HL60 cells²⁹ (from D. Simmons). Both hCX5.1 and hCX5.36 were isolated several times and after restriction mapping and partial sequencing of both clones, the complete nucleotide sequence of hCX5.36 was determined by primer walking using double-stranded dideoxy-sequencing. The GenBank accession number for hCX5.36 (CB2) is X74328.

*a***HCX5.36**

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CAGGTCCTGGGAGAGGACAGAAAACACTGGACTCCTCAGCCCCGGCAGCTCCAGTGCCAGCCACCCACACACAAACCAAGCCTT 90
                                     MetGluGluCysTrpValThrGluIleAlaAsnGlySerLysAspGlyLeuAsp
CTAGACAAGCTCAGTGAATCTGAGGGCCACCCCATGGAGGAATGCTGGTGACAGAGATAGCCAATGGCTCCAAGGATGGCTGGAT 180
SerAsnProMetLysAspTyrMetIleLeuSerGlyProGlnLysThrAlaValAlaValLeuCysThrLeuLeuGlyLeuLeuSerAla
TCCAACCCCTATGAGGATTACATGATCTGAGTGGTCCCGAGAGACAGCTGTTGCTGTGTGTGCACTCTCTGGGCTGCTAAGTGCC 270
LeuGluAsnValAlaValLeuTyrLeuIleLeuSerSerHisGlnLeuArgArgLysProSerTyrLeuPheIleGlySerLeuAlaGly
CTGGAGAACCTGGCTGTGCTCTATCTGATCTGCTCCACCACTCCGCGGAAGCCCTCATACCTGAGTGTGAGCTTGGCAGCTGGCTGGG 360
AlaAspPheLeuAlaSerValValPheAlaCysSerPheValAlaAsnPheHisValPheHisGlyValAspSerLysAlaValPheLeuLeu
GCTGACTTCTGGCCAGTGTGGTCTTTGCTGAGCTTTGTGAATTTCCATGTTTCCATGGTGTGGATTCCAAGGCTGCTTCTGCTGCTG 450
LysIleGlySerValThrMetThrPheThrAlaSerValGlySerLeuLeuLeuThrAlaIleAspArgTyrLeuCysLeuArgTyrPro
AAGATTGGCAGCTGACTATGACCTTCACAGCCTCTGTGGTAGCCCTCTGCTGACCGCATTGACCGATACCTCTGGCTGCTATCCA 540
ProSerTyrLysAlaLeuLeuThrArgGlyArgGlyLeuValThrLeuGlyIleMetTrpValLeuSerAlaLeuValSerTyrLeuPro
CCTTCTCAAAAGCTCTGCTCACCCGTCAGGCTGAGTGTGGTCTTCCACTGATCCCAATGACTACCTGCTGAGCTGGCTGCTGCTGCTG 630
LeuMetGlyTrpThrCysCysProArgProCysSerGluLeuPheProLeuIleProAsnAspTyrLeuLeuSerTrpLeuLeuPheIle
CTCATGGATGGACTTGTGCTGCCAGGCCCTGCTCTGAGCTTTTCCACTGATCCCAATGACTACCTGCTGAGCTGGCTGCTGCTGCTGCTG 720
AlaPheLeuPheSerGlyIleIleTyrThrTyrGlyHisValLeuTrpLysAlaHisGlnHisValAlaSerLeuSerGlyHisGlnAsp
GCCTTCTCTTTCCGGAATCATCTACACCTATGGCATGTCTCTGGAAGGCCCATCAGCATGTGGCCAGCTTGTCTGGCCACCAAGGAC 810
ArgGlnValProGlyMetAlaArgMetArgLeuAspValArgLeuAlaLysThrLeuGlyLeuValLeuAlaValLeuLeuIleCysTrp
AGGCAGGTGCCAGGAATGGCCGAATGAGGCTGGATGTGAGGTTGCCAAGACCCATAGGGCTAGTGTGGCTGTGCTCTCATCTGTTGG 900
PheProValLeuAlaLeuMetAlaHisSerLeuAlaThrThrLeuSerAspGlnValLysLysAlaPheAlaPheCysSerMetLeuCys
TTCCAGTGCTGGCCCTCATGGCCACAGCCTGGCCACTACGCTCAGTGACAGGTCAGAGAGCCCTTGTCTTCTGCTCCATGCTGTGCT 990
LeuIleAsnSerMetValAsnProValIleTyrAlaLeuArgSerGlyGluIleArgSerSerAlaHisHisCysLeuAlaHisTrpLys
CTCATCACTCCATGGTCAACCTGTCTATGCTCTACGGAGTGGAGAGATCCGCTCTCTGCCCATCACTGCTGGCTGCTGCTGCTGCTG 1080
LysCysValArgGlyLeuGlySerGluAlaLysGluGluAlaProArgSerSerValThrGluThrGluAlaAspGlyLysIleThrPro
AAGTGTGTGAGGGCCCTGGGTGAGAGCAAAAGAAGAGCCCGAGATCCTCAGTCACCGAGACAGAGGCTGATGGGAAATCACTCCG 1170
TrpProAspSerArgAspLeuAspLeuSerAspCys***
TGCCAGATTCAGAGATCTAGACCTCTCTGATTGCTGATGAGGCTCTTCCCAATTTAAACAACCTCAAGTCAGAAATCAGTTCCTCTCC 1260
TGGAAGAGAGAGAGGGGTCTTGGCACTCTCTTACTTAAACAGTCCAGACACCTAGACACGACCCCTTTTGTCTGATGAGTGTG 1350
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CCAGGCCAGCAATGAGGACTTGGGAGAAATCTGAGAAGATGGGTGTCTCTTGGGAAGTCAGGATATCAGATGGGATGACATCCA 1620
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GGACTATGCTATGATGAGGATTAAGGTGTGACTTGCCTCTTTCAGAGATAATGACAAGCCTTCAAAAAAAAAAAAAA 1790

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HCX5.1:

....TATGCTATGATGAGGATTAAGGTGTGACTTGCCTCTTTCAGAGATAATGACAAGCCTTCAGTTGGGTCATCTGTTGTTTG.

b

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hCB-R      MKSILDGLADTTFTTITDILLYVGSNDIQYEDIKGDMSKLGYPFQ
hCX5       MEECWVTEIANGSKDGLDSN---- 20
hCB-R      KFPLTSFRGSPFQEKMTAGDNQPLVPADQVNIITFYNSLSFKENEINI
-----PMKDYMITLSPGKTAVAVUCLLGLSALENVAVLYLTSSHQ 63
OQGENFMDEICFIMNPSQOLATAVLSLTGFTVLENLLVCVILHSRS
LRRKPSYLFISLAGADFDASVFAFSVNFHVFHGVDSKAVFLRIGSM 113
LRCPSYFIFISLAVLADLVSVITVYSHIDFHFVRKDSRNVELFLGGM
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IASETASVGSLLTAIDRYLTSIHRELAKYRIVTEPKAMVAFCLMNTIATV
VSYLPDGNICPCRP--ESELFLIPNDVILSLLLFIAHDFSGDITVYGH 211
IAVLPLIGNICEKLGQSCSTEDFIDETVIMFTGVTSVLLFRIMAMNY
VLWKAHRAVASL-----SGHQDRV--FGMARMLDMLAKT 246
ILWKAHSHAVRMIOGRTGKSIITHTSEGGKVQVTRDQARM--DILAKT
LGLMDVLLTQVFLVLAHSLATLSDQVKAFAFCSMLCCLINSVWNP 296
LMVLVLLTQVFLVLAHSLATLSDQVKAFAFCSMLCCLINSVWNP
VIYALRSGEIRSS-----AHICLAHWKKCVRLGGS 326
IYIALRSKDLHAFRSMFPSCGTAQPLDNSMGDSCLHKHANNASVHR
EAKKEAPRSSTTETADGKTTPWPSRDLLSDC 360
IAESCKISTV-----KIAKVTMSVSTDSAEAL

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of other tissues (Fig. 3b). In particular, the rCX5 transcript is not detected in brain, even though the 6 kb mRNA encoding the rat CB-R can be readily detected in the same sample. The expression pattern of the CB-R gene corresponds well to the distribution of binding sites for cannabinoids in the brain^{6,18}. But it is possible that CX5 is expressed in a subset of these sites and its expression level is too low to be detected in total brain RNA. To investigate this possibility horizontal sections of rat brain were probed by *in situ* hybridization with labelled oligonucleotides corresponding to rat CB-R and to rCX5 (Fig. 4). As previously reported, the brain receptor has a widespread distribution with high levels of expression in the cortex, hippocampus, striatum and cerebellum. When adjacent sections were probed for rCX5, no expression could be detected in these, or any other, regions. The rCX5 oligonucleotide does, however, hybridize to localized regions of the spleen (Fig. 4b, c). The expression appears concentrated in the marginal zones found around the periarteriolar lymphoid sheaths. The expression of hCX5 in HL60 cells differentiated along the myeloid lineage implies that this expression is likely to be in macrophages. To confirm this, splenic macrophages/monocytes were purified using cell sorting

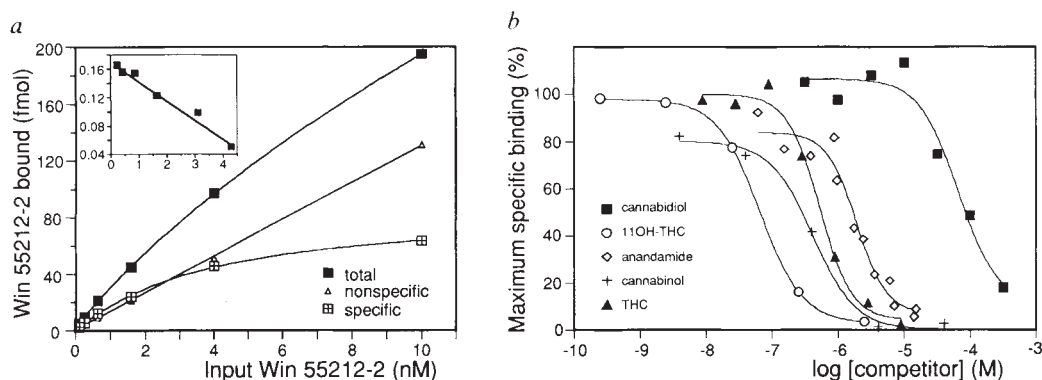


FIG. 2 Binding of cannabinoids to the receptor encoded by hCX5.36. *a*, Binding of [3 H]Win 55212-2 to membranes from COS cells transfected with an expression plasmid SC36, that contains the hCX5.36 cDNA. The inset plot shows the specific binding presented as bound/free against [bound] ($\text{M} \times 10^{-10}$). *b*, Displacement by cold cannabinoids of [3 H]CP55,940, or of [3 H]Win 55212-2, bound to membranes from COS cells transfected with SC36.

METHODS. Plasmid SC36 is hCX5.36 inserted into the vector CDM8³⁰. 72 h after transfection, cells were Dounce homogenized and the membranes pelleted from the post-nuclear supernatant at 90,000g for 20 min, washed and then resuspended in 50 mM Tris-HCl pH 7.4, 3 mM MgCl_2 , 1 mM EDTA and stored in liquid N_2 . Binding of [3 H]Win 55212-2 (49.6 Ci mmol^{-1} ; New England Nuclear) to membranes (40 μg membrane protein per 150 μl reaction), was determined essentially as

described, except that siliconized 1.5 ml polypropylene tubes were used for the binding reactions and 5% ethanol, 5% Triton X-100 was used to solubilize the membrane pellets⁹. Nonspecific binding was measured in the presence of 10 μM Δ^9 -THC, and data points shown are means of duplicates (average duplicate's difference 4.3%). Displacement by cold cannabinoids (Sigma) was determined using 1.0 nM [3 H]CP55,940 (107 Ci mol^{-1} ; New England Nuclear), or for anandamide (provided by R. Mechoulam) and cannabidiol, 1.0 nM [3 H]Win55212-2, although similar results were obtained for all competitors with both hot ligands (not shown). The anandamide displacement curve comprises data from two separate experiments. All data points are means of duplicates and all experiments were repeated at least twice. Inhibition constants (K_i) in nM: 11-OH- Δ^9 -THC, 40 $\pm$ 1.5; Δ^9 -THC, 320 $\pm$ 80; cannabinalol, 250 $\pm$ 80; cannabidiol, 38,000 $\pm$ 18,000.

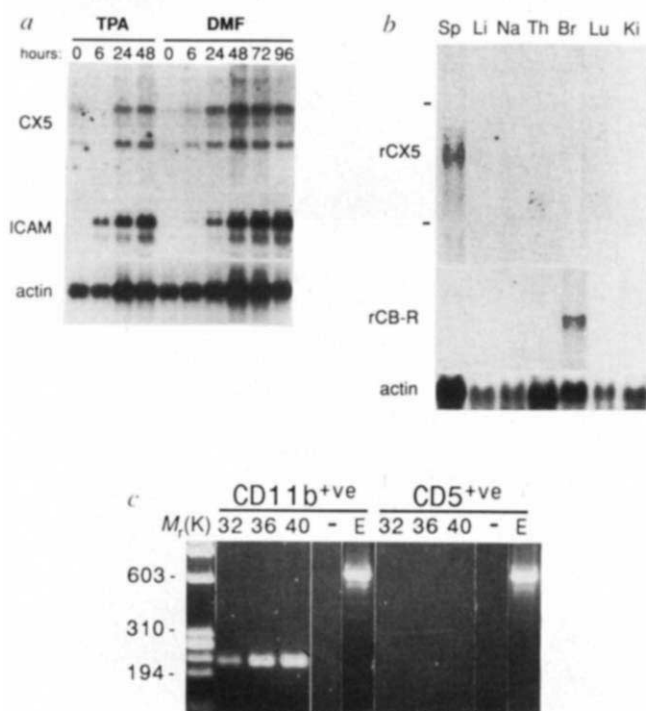


FIG. 3 Expression of CX5 transcripts in HL60 cells and in rat tissues. *a*, Northern blots of RNA from HL60 cells induced with either 20 ng ml^{-1} TPA or with 0.7% DMF, probed with either hCX5, or with ICAM-1 to follow induction. The hCX5 blot was then reprobed for γ -actin. *b*, Northern blot of RNA from various rat tissues probed with a rat homologue of CX5 (SP, spleen; Li, liver; Na, nasal epithelium; Th, thymus; Br, brain; Lu, lung; Ki, kidney). The blot was then reprobed with the rat cannabinoid receptor (rCB-R) and then with actin. *c*, PCR analysis of rCX5 expression in sorted rat spleenocytes. cDNA from rat spleenocytes sorted using antibodies against CD11b for macrophages/monocytes, or CD5 for T-

cells, was amplified with primers specific for rCX5, with products being removed after the indicated number of cycles (32,36,40). To demonstrate that the rCX5 signal derives from mRNA, cDNA reactions without added reverse transcriptase were amplified for 40 cycles (—) and, as a positive control, cDNAs were amplified with primers specific for elongation factor 1 α (E).

METHODS. Total RNA was isolated from HL60 cells by lysis in guanidinium/LiCl, and 7.5 μg per sample was separated on a 1.2% agarose/4% formaldehyde gel, transferred to nylon (Hybond-N, Amersham) and ultraviolet cross-linked. Parallel blots were probed at 42 $^{\circ}\text{C}$ in 5 $\times$ SSPE/50% formamide/100 $\mu\text{g/ml}$ salmon sperm DNA/5 $\times$ Denhardt's/0.1% SDS with either CX5 or ICAM-1²⁹ labelled with ^{32}P by random-priming (Pharmacia). After washing with 1 $\times$ SSC at 42 $^{\circ}\text{C}$, the blot was exposed to Kodak XAR with an intensifying screen, ICAM, 8 days; hCX5, 10 days). The blots were then stripped according to the manufacturer's instructions and reprobed with human γ -actin, exposure 6 h. The fall in expression at the 6 h time point in TPA was reproducible. A rat homologue of CX5 was cloned from genomic DNA by PCR using primers. GGGCTCGAGGTNRAYTTYCAYGNTT and GAGGGATCCATNSWRCARAANGCRAA that encode sequences in hCX5 which are also found in the cannabinoid receptor but not in other G-protein-coupled receptors (VNFHV (91–96) and FAFCSM (279–284)). Cloning of PCR products of 600–650 bp produced primarily the rat cannabinoid receptor or a sequence with 88% homology to hCX5, which was termed rCX5 (S.M. unpublished observations). Total RNA extracted by guanidinium lysis from various rat tissues (5–10 μg per lane) was blotted and probed as before with rCX5, rat cannabinoid receptor and γ -actin and then exposed using a phospho-imager (Molecular Dynamics) for rCX5 (9 h), CB-R (4 h) or XAR film (12 h) for actin. For PCR analysis, rat spleenocytes were separated by FACS using MRC OX-42 (CD11b)³¹ or MRC OX-19 (CD5)³² (Serotec). Cytoplasmic RNA prepared from 3×10^5 cells by detergent lysis, (20 μg glycogen added as carrier) was treated with ribonuclease-free DNase (0.5 unit for 10 min; RQ1, Promega), phenol extracted, ethanol precipitated, resuspended in reverse transcriptase buffer with random primers, divided in two and MMLV reverse transcriptase was added to one set (GIBCO). After 60 min at 37 $^{\circ}\text{C}$, PCR amplification was done with the primers TTTCACGGTGTGGACTCC and TAGGTAGGAGATCAAGCG (rCX5, 214 bp product) or GAAATGCACCATGAAGT and TTACGATGCATTGTTATC (EF-1 α , 645 bp product from spliced transcript³³) using 94 $^{\circ}\text{C}$, 1 min 54 $^{\circ}\text{C}$, 1 min, 72 $^{\circ}\text{C}$ 1 min with the manufacturer's buffer (Promega).

and the expression of CX5 examined using PCR. Expression was detected in the macrophage/monocyte population but not in the CD5-positive population used as a control.

The marginal zone is the site through which blood-borne cells and antigens enter the spleen, and the marginal zone macrophages comprise a distinct population of highly phagocytic cells thought to play a role in both digesting and processing bacterial

antigens and in directing lymphocyte recirculation^{19,21}. We are currently preparing antibodies specific for CX5 to investigate its cellular distribution in more detail, but the *in situ* distribution suggests that its expression is concentrated in these marginal zone macrophages. The *in vivo* function of CX5 is presumably to transduce a signal through a G-protein in response to an endogenous ligand, although we do not yet have direct evidence

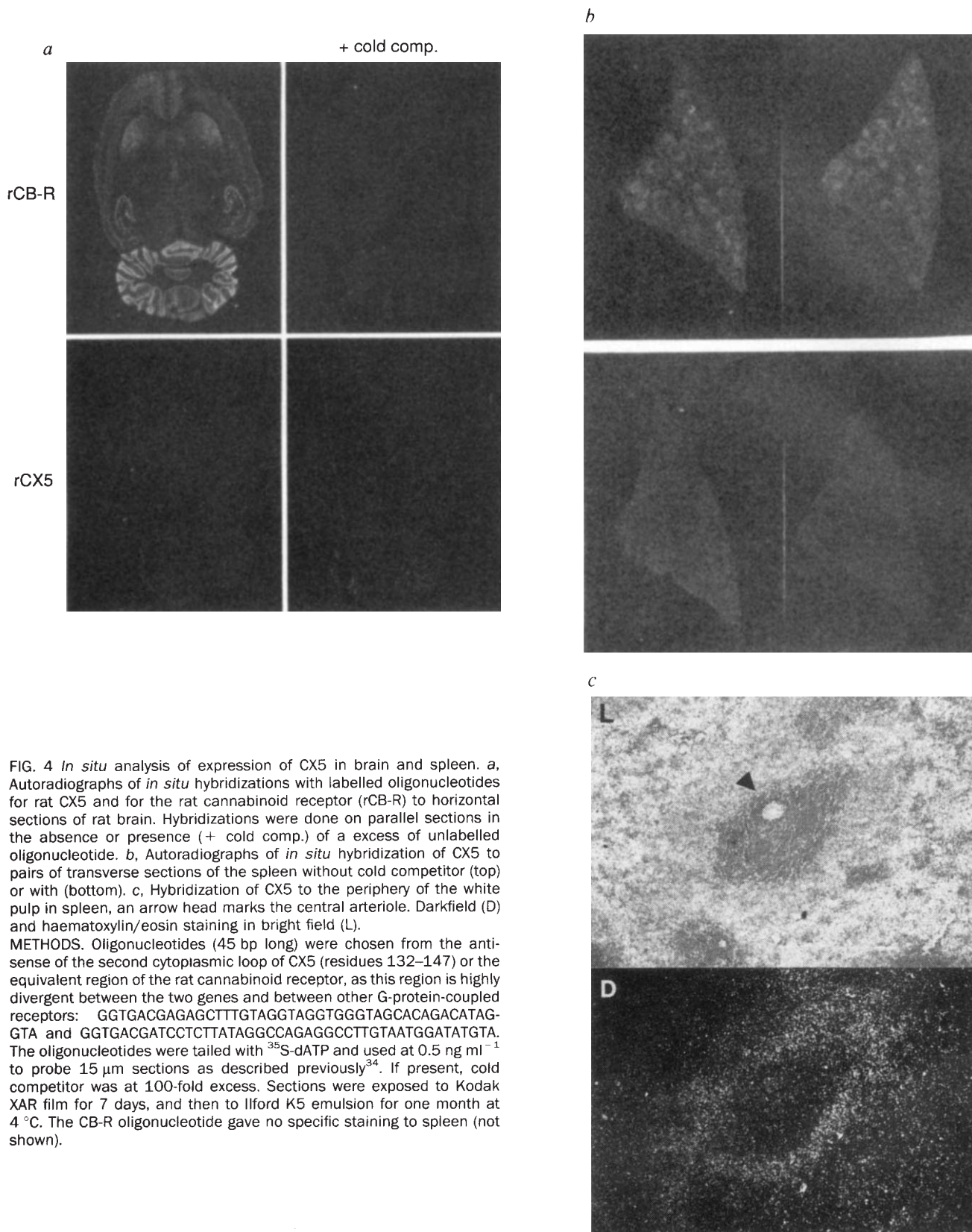


FIG. 4 *In situ* analysis of expression of CX5 in brain and spleen. a, Autoradiographs of *in situ* hybridizations with labelled oligonucleotides for rat CX5 and for the rat cannabinoid receptor (rCB-R) to horizontal sections of rat brain. Hybridizations were done on parallel sections in the absence or presence (+ cold comp.) of a excess of unlabelled oligonucleotide. b, Autoradiographs of *in situ* hybridization of CX5 to pairs of transverse sections of the spleen without cold competitor (top) or with (bottom). c, Hybridization of CX5 to the periphery of the white pulp in spleen, an arrow head marks the central arteriole. Darkfield (D) and haematoxylin/eosin staining in bright field (L).

METHODS. Oligonucleotides (45 bp long) were chosen from the anti-sense of the second cytoplasmic loop of CX5 (residues 132–147) or the equivalent region of the rat cannabinoid receptor, as this region is highly divergent between the two genes and between other G-protein-coupled receptors: GGTGACGAGAGCTTTGTAGGTAGGTGGGTAGCACAGACATAG-GTA and GGTGACGATCCTCTTATAGGCCAGAGGCCTTGTAAATGGATATGTA. The oligonucleotides were tailed with ³⁵S-dATP and used at 0.5 ng ml⁻¹ to probe 15 µm sections as described previously³⁴. If present, cold competitor was at 100-fold excess. Sections were exposed to Kodak XAR film for 7 days, and then to Ilford K5 emulsion for one month at 4 °C. The CB-R oligonucleotide gave no specific staining to spleen (not shown).

for such coupling. Analysis of other seven spanning receptors has suggested that basic, amphipathic helices at either end of the third cytoplasmic loop are involved in G-protein coupling and appropriate residues are found in these positions both in the CX5 sequence and in the brain receptor, which has been shown to be G-protein coupled^{6,22}.

There are many reports of cannabinoids exerting suppressive effects on various cells of the immune system, including macrophages²³⁻²⁵, although the significance of some of these observations has been questioned because of the high doses of drug used²⁶. But the location of the CX5 receptor, and its distinct structure from the brain receptor, strongly suggest that the endogenous ligand for these receptors will have an immuno-modulatory role in addition to its neuronal function. Anandamide has been recently identified as a candidate ligand for the cannabinoid receptor¹⁶ and this compound also binds to the CX5 receptor, although with an apparent affinity 30-fold less than that reported for the brain receptor. Anandamide is able to cross the blood brain barrier rapidly²⁷ but worthwhile speculation as to its function, and possible interactions between the neural and immunological systems, will require the identification of all the sources of this intriguing molecule. Furthermore, the question of further potential ligands from brain remains to be resolved¹⁶. Even so the existence of the CX5 receptor does have further implications. G-protein-coupled receptors are highly conserved throughout evolution¹², and yet the sequence of CX5 is considerably divergent from that of CB-R. Of the 162 residues in transmembrane sections of the human CB-R, three are different in rat CB-R, but 68 are different in human CX5. This suggests that the two receptors did not diverge recently and furthermore it suggests that it should be possible to identify receptor-specific cannabinoids. The fact that cannabinol appears to have a higher relative affinity for the CX5 receptor than for the brain receptor, may provide the basis for identifying such a ligand for the CX5 receptor. We suggest that in future the two receptors be distinguished by calling the brain receptor CB1 and the CX5 receptor CB2. It has been proposed that the peripheral effects of cannabinoids are either indirect effects of central actions, or reflect interactions with non-receptor proteins such as lipoxygenases^{1,8}. It is clearly possible that some of these peripheral effects are in fact mediated through the CB2 receptor and it will be interesting to determine the activities of any cannabinoids specific for this receptor. □

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Cloning and gene defects in microsomal triglyceride transfer protein associated with abetalipoproteinaemia

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THE microsomal triglyceride transfer protein (MTP), which catalyses the transport of triglyceride, cholesteryl ester and phospholipid between phospholipid surfaces, is a heterodimer composed of the multifunctional protein, protein disulphide isomerase, and a unique large subunit with an apparent M_r of 88K (refs 1-3). It is isolated as a soluble protein from the lumen of the microsomal fraction of liver and intestine⁴. The large subunit of MTP was not detectable in four unrelated subjects with abetalipoproteinaemia⁵, a rare autosomal recessive disease characterized by a defect in the assembly or secretion of plasma lipoproteins that contain apolipoprotein B (ref. 6). We report here the isolation and sequencing of complementary DNA encoding the large subunit of MTP. A comparison of this sequence to corresponding genomic sequences from two abetalipoproteinaemic subjects revealed a homozygous frameshift mutation in one subject and a homozygous nonsense mutation in the other. The results indicate that a defect in the gene for the large subunit of MTP is the proximal cause of abetalipoproteinaemia in these two subjects, and that MTP is required for the secretion of plasma lipoproteins that contain apolipoprotein B.

Based on the sequence of 1 of 10 peptides isolated from the large subunit of MTP (Fig. 1), a 20-base, 32-fold degenerate oligonucleotide probe was designed and used to screen a λ gt10, bovine small intestine cDNA library. Overlapping bovine clones

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Complementary DNA libraries generated from human liver and human small intestine messenger RNA were screened with a bovine cDNA probe. The entire coding sequence for the large subunit of MTP was obtained independently from each library.

[illegible]

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and no difference in the cDNA sequences was observed (Fig. 1). Comparison of the bovine and human cDNAs revealed 88% nucleotide identity in the coding region. Sequence analysis of the human gene (our manuscript in preparation) revealed that the 18 A residues located at the 3' end of the human cDNA are encoded by genomic DNA. Polyadenylation of the message may occur at one of two AATAAA consensus signals⁸ found 550 and 676 bases 3' of the 18 base stretch of A in the human genomic DNA. The predicted translation product of the large subunit of human MTP contains 894 amino acids. Cleavage between the signal peptide and the mature protein is predicted to occur between amino acid 18 and 19 of the human protein⁹. The mature protein has a predicted M_r of 97K. There is 86% identity between the deduced amino-acid sequence of bovine and human MTP large subunit. Allowing for conservative amino-acid substitutions, there is 94% homology. There was no obvious homology between these sequences and that of any protein in available sequence data banks.

To confirm that the cDNA encodes the microsomal triglyceride transfer protein, the full coding sequence for the large subunit of MTP was subcloned into an expression vector and was transiently expressed in transformed human skin fibroblasts¹⁰. Extracts from the transfected cells showed substantial triglyceride transfer activity above background levels (Fig. 2). In addition, northern blot analysis of poly(A)⁺ RNA from available human tissues (Fig. 2) revealed message levels that are consistent with the known tissue distribution of MTP activity⁴.

An intestinal biopsy was obtained from a 39-year-old abetalipoproteinaemic female offspring of a consanguineous mating. We had previously demonstrated the absence of MTP activity

and protein in this subject⁵. Total RNA was isolated and reverse transcribed into first-strand cDNA. The cDNA encoding the large subunit of MTP was amplified by PCR and sequenced directly. Three independent PCR products revealed a cytosine deletion at base 215 (Fig. 3). The frameshift mutation leads to a premature stop codon 21 bases downstream and a predicted translation product of 78 amino acids. To determine whether both alleles of the gene encoding MTP contain the frameshift mutation, the 187 base-pair exon (our manuscript in preparation) that encodes the mutation was amplified from the subject's genomic DNA and sequenced directly. A single sequence that contained the frameshift mutation was observed, indicating that both alleles contain the defect. The mutation was also confirmed by sequencing eight plasmid clones of the PCR product.

A genomic library was constructed from the DNA of a 14-year-old abetalipoproteinaemic female who lacks MTP⁵ and clones encoding the large subunit of MTP were isolated. Sequence analysis revealed a C to T mutation corresponding to base 1,783 of the cDNA. This changes an Arg codon to a premature stop codon. A truncated translation product of 594 amino acids is predicted. This nonsense mutation also eliminates a *TaqI* restriction endonuclease site. To determine if the subject is homozygous for this mutation, genomic DNA was digested by *TaqI* and analysed by Southern hybridization. The normal exon encoding base 1,783 was digested into two fragments by *TaqI* (Fig. 4). DNA from the abetalipoproteinaemic subject (proband) was not digested at this site, indicating both alleles contain the defect. The parents displayed the restriction pattern of both the normal and mutant alleles.

The mutations identified in the two abetalipoproteinaemic

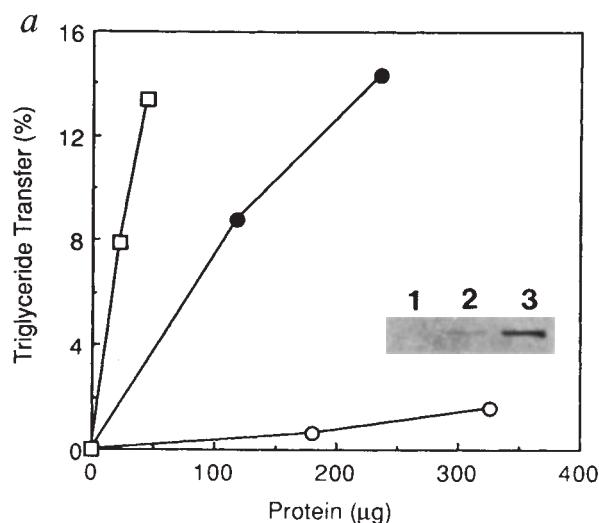
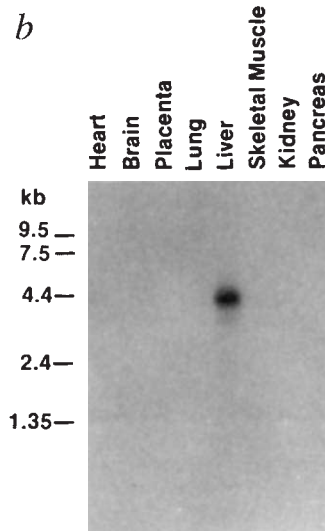


FIG. 2 a, Expression of human MTP in transformed human skin fibroblasts (1508T)¹⁰. Transfer activity was measured in extracts of 1508T cells transfected with either pcDNA/Neo (negative control, open circles) or pcDNA/MTP (solid circles). For comparison, transfer activity was also measured in extracts of HepG2 cells (squares). Activity is expressed as the percent of radiolabelled triglyceride transferred from donor vesicles to acceptor vesicles as a function of cell protein. Aliquots (20 μ g cell protein) of the same cell extracts used for activity measurements were analysed for expression of MTP by western blot (inset) with an antibody directed against the large subunit of MTP. Lanes 1–3 are extracts from 1508T cells transfected with pcDNA/Neo, 1508T cells transfected with pcDNA/MTP, and HepG2 cells, respectively. b, A northern blot containing 2 μ g poly(A)⁺ RNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas was hybridized to a cDNA probe for the large subunit of MTP. A 4.4-kb band in the liver lane was apparent. RNA from human intestine was not available for northern blot analysis, however, the presence of MTP mRNA in intestine was evident from the cDNA clones obtained from the human intestinal library. In addition, MTP mRNA was readily detectable in hamster intestine (not shown).



METHODS. a, A 3.2-kb fragment containing the entire coding sequence for human MTP, extending from nucleotide -64 to 3,138 was subcloned into plasmid pcDNA/Neo (Invitrogen) to yield plasmid pcDNA/MTP. Transfection mixtures were made by dissolving 50 μ g plasmid in 1.5 ml serum-free DMEM. This was added dropwise to a solution of 120 μ l lipofectin reagent (Bethesda Research Laboratories) dissolved in 1.5 ml serum-free DMEM. After a 15-min incubation at room temperature, the transfection mixtures were added to 100-mm dishes of 1508T cultures (plated at 25% confluency 24 h before transfection) containing 7 ml serum-free DMEM. Twenty-four h later the transfection mixtures were removed and 10 ml fresh DMEM containing 10% fetal bovine serum was added for an additional 24 h. Cells were scraped from the dishes and washed with phosphate-buffered saline. Cell extracts, MTP activity measurements, and western blot analyses were as described previously⁵. b, A prepared northern blot was purchased from Clontech. Each lane contained 2 μ g poly(A)⁺ from the indicated tissue. The blot was hybridized to a 2.4-kb *EcoRI* bovine cDNA probe for the large subunit of MTP as described in Fig. 1.

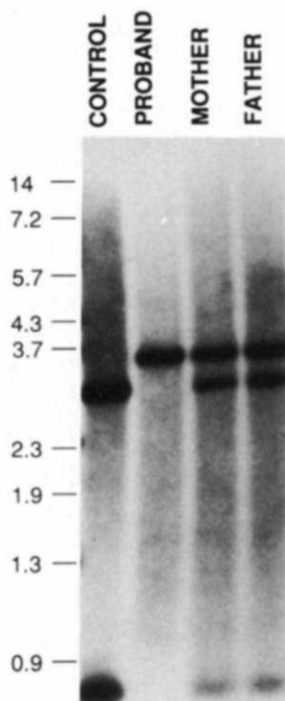
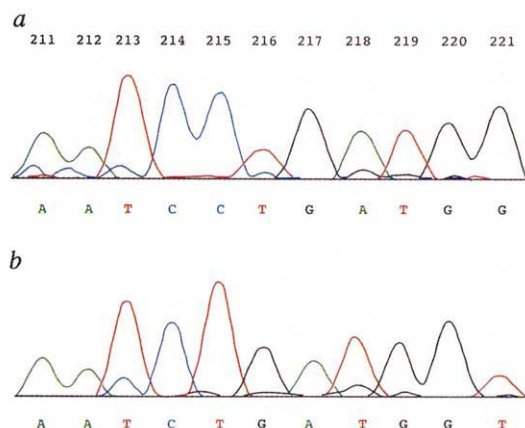


FIG. 4 The loss of *TaqI* restriction endonuclease site in an abetalipoproteinaemic patient. Genomic DNA was isolated from a control subject, the proband, and the proband's mother and father, digested with *TaqI*, and Southern blotted. The filter was hybridized to a PCR probe of the exon containing the mutation. The presence of the restriction site in the control DNA is indicated by the two hybridizing fragments at 3.0 and 0.6 kb. The deletion of the *TaqI* site in both alleles of the abetalipoproteinaemic subject's genomic DNA is demonstrated by the absence of these bands and the presence of a single larger hybridizing band at 3.6 kb. The presence of all three hybridizing bands in the proband's parents' DNA confirms that both parents are heterozygous for the mutation.

METHODS. Blood (6 ml) from a control subject, the proband, and the proband's parents was drawn into heparin tubes and genomic DNA was prepared from each sample with a Qiagen Blood DNA kit following the manufacturer's protocol. Each DNA (10 µg) was digested with *TaqI*, electrophoresed on a 1.0% agarose gel and blotted to nitrocellulose. A 302-bp probe comprising the exon (from bp 1,770 to 1,867 of the cDNA) which contains the mutation and flanking intron sequences, was generated by PCR of DNA with primers designed from intron sequences (5'-ATTGGCTCCTCTTTT-3' and 5'-GGGACTACCTGATGACACA-3'). The filter was probed at 65 °C using conditions described in Fig. 1. The filter was washed at 65 °C in 1 × SSC, 0.1% SDS.

FIG. 3 Frameshift mutation in the gene encoding the large subunit of MTP in an abetalipoproteinaemic subject. Normal (a) and mutated (b) sequences from base 211 to base 221 are compared. The sequence obtained from the abetalipoproteinaemic subject exhibits a cytosine deletion at base 215. Identical sequence was obtained from PCR-amplified RNA or DNA from the subject.

METHODS. Total RNA was isolated from an intestinal biopsy by the RNAzolB method (CinnaBiotex Labs). RNA (50 ng) was reverse-transcribed into first-strand cDNA using random hexamer primers and reagents supplied by Perkin-Elmer-Cetus. PCR reactions were as described in the legend to Fig. 1 except 50 cycles were conducted. Primer sets amplified DNA between bases -8 and 605, -8 and 767, and 14 and 960 (see Fig. 1). Amplified DNA was gel purified using GeneClean. A portion of the DNA was sequenced directly. The remaining DNA was subcloned into pUC18 plasmid and multiple clones were sequenced. Genomic DNA, isolated from a second biopsy, was heated to 95 °C for 5 min and added to a PCR as described above using primers designed from the sequences of introns flanking the exon (from bp 62 to 249 of the cDNA) containing the mutation. The forward and reverse primers were 5'-CCCTTACAATGAAACTGG-3' and 5'-GGTAC-ACTTCTCCAAAACTT-3', respectively. Amplification was at 97 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min for 3 cycles. The denaturation temperature was lowered to 94 °C for 32 additional cycles. The PCR products were purified and sequenced as described above.

subjects explain the absence of MTP activity and protein, and indicate that a defect in the gene for the large subunit of MTP is the proximal cause of abetalipoproteinaemia in these subjects. These results also demonstrate that MTP is required for the assembly of plasma lipoproteins that contain apolipoprotein B (apoB). The mechanism by which apoB and lipid associate to form a mature lipoprotein particle in the liver or intestine is not fully understood. Newly synthesized apoB may be degraded or secreted depending upon the availability of cholesteryl ester¹¹ or triglyceride^{12,13}. In the presence of neutral lipid, apoB is protected from proteolytic degradation and forms a mature lipoprotein particle which is secreted. When cholesteryl ester or triglyceride availability is limited, apoB is degraded in a pre-Golgi compartment¹⁴⁻¹⁶. The results of this study indicate that MTP is necessary for the assembly of apoB particles which are protected from proteolytic degradation and are destined for secretion. An MTP-mediated addition of lipid to apoB early in the assembly process may play a key role in the stabilization of apoB. MTP may also participate in the transfer of additional lipid to apoB particles at stages distal to the sorting point between degradation and secretion.

As a result of the well established relationship between elevated plasma cholesterol levels and premature coronary heart disease¹⁷, considerable research has focused on identifying new approaches to lower plasma lipid levels. The results of this and previous investigations indicate that: (1) plasma chylomicrons, very low-density, and low-density lipoproteins are absent in abetalipoproteinaemic subjects⁶; (2) the defect in abetalipoproteinaemia is specific for lipoprotein assembly in that complications of this disease are secondary to deficiencies of fat soluble vitamins which are transported on apoB-containing lipoproteins⁶; and (3) defects in MTP can cause abetalipoproteinaemia. Thus, we propose that inhibition of MTP will provide a specific mechanism for lowering plasma cholesterol and triglyceride levels. □

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A mouse homologue of the *Drosophila* tumour-suppressor gene *l(2)gl* controlled by Hox-C8 in vivo

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THE homeobox is a 183-base-pair DNA sequence originally found in *Drosophila* segmentation and homeotic genes^{1,2}. In *Drosophila*, homeotic genes are clustered in the *Antennapedia* and *Bithorax* complexes, collectively called the homeotic gene complex (HOM-C)³. In the mouse genome, about 40 homeobox genes (*Hox*) are clustered in four chromosomal regions (*Hox A* to *D*). The *Hox* genes are arranged in the same order and have the same antero-posterior pattern of expression as their structural homologue in the HOM-C^{4,5}, suggesting that they control mouse pattern formation in the same way that HOM-C members do in *Drosophila*. Homeobox gene products are believed to be transcription factors that regulate expression of target genes. A few candidate target genes have been identified in *Drosophila* by various approaches⁶ but the *Hox* gene targets are poorly understood, mostly because of limitations in the available approaches. Here we identify several candidate *Hox* gene targets, including a mouse homologue of the *Drosophila* tumour-suppressor gene *l(2)gl*, by immunopurification of DNA sequences bound to a Hox protein in native chromatin.

To identify *Hox* gene targets we first isolated the DNA sequences bound by a Hox protein in native chromatin by immunochemical means, and then identified the gene in the isolated sequence. The antibodies used were raised against a bacterially synthesized Hox-C8 (Hox 3.1) protein, and gave immunohistochemical staining patterns on sections of mouse embryos almost identical to those previously reported⁷.

To isolate Hox-C8 target sequences from spinal cord, soluble nucleoprotein complexes were prepared from paraformaldehyde-fixed mouse spinal cords by sonication, passed through the anti-Hox-C8 antibody affinity column, and treated with protease. After the immunoenriched DNA had been digested with *Hae*III and cloned into λ gt10 vector, about 2×10^4 independent clones were obtained. Cloning efficiency from the immunoenriched DNA was about one-tenth that of the unfixed genomic DNA. To assess the enrichment of the Hox-C8-bound sequences, we asked whether the *Hox-A7* (*Hox 1.1*) 5' flanking sequence is significantly represented in the library, because homeobox genes themselves are potential targets of homeobox gene products^{8,10}. A 5.0 kilobase (kb) restriction fragment corresponding to the 5'

flanking region of *Hox-A7* hybridized to 80 independent clones among the 2×10^4 library clones. Because only one out of 5×10^3 random genomic clones is expected to hybridize with a 5.0 kb unique genomic sequence, the 5' flanking sequence of the *Hox-A7* gene in mouse spinal cord and that *Hox-A7* is a candidate target gene of *Hox-C8*. *Hox-A7* 5' flanking sequence was

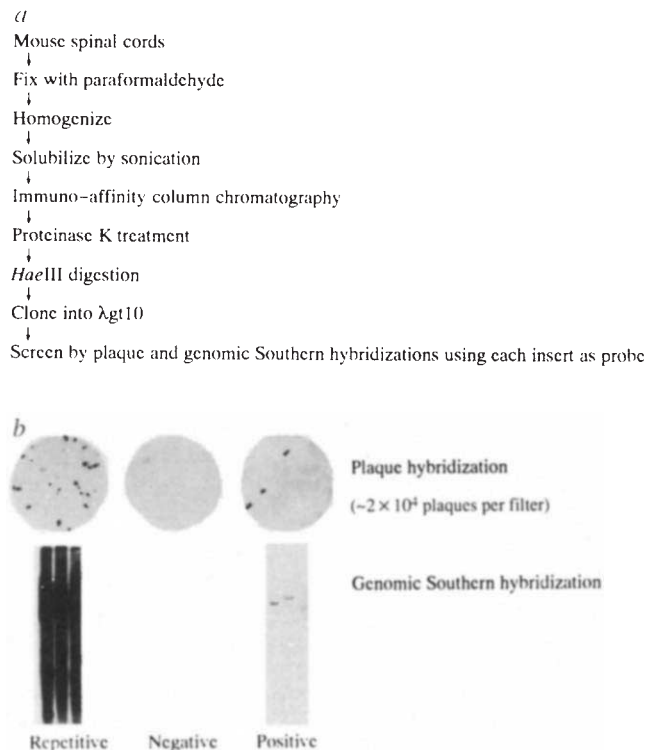


FIG. 1 The strategy for isolation of the *Hox-C8* target sequence, a, Procedure for immunoenrichment. b, Three typical examples of the plaque—and genomic Southern hybridizations. Left, Labelled insert hybridized with multiple plaques in a filter replica of the immuno-enriched clones and also hybridized with a smear of restricted fragments on a genomic Southern blot which indicates inclusion of repetitive sequences in the insert. Middle, Labelled insert did not hybridize with any plaques of a filter replica. Right, Labelled insert hybridized with multiple plaques in a filter replica and hybridized with a unique restricted fragment on a genomic Southern blot.

METHODS. Antibodies: The *Sac*I–*Xho*I fragment of the first exon of *Hox-C8* was prepared from a mouse genomic clone mg51 and cloned into T7 expression plasmid pET3B²². Preparation of antisera and affinity purification of anti-Hox-C8 antibodies were as described²³. Immunopurification: Spinal cords of five ICR mice were incubated in 10 mM Na-phosphate buffer pH 7.0, 150 mM NaCl containing 4% paraformaldehyde, at 0 °C, overnight. Fixed spinal cords were homogenized and washed with 10 mM Na-phosphate buffer pH 7.0, 150 mM NaCl. The nucleoprotein complexes were solubilized by sonication. After centrifugation at 10,000g for 15 min, the supernatant was loaded on the antibody-bound Sepharose CL-4B affinity column. The column was washed with 10 mM Na-phosphate buffer pH 7.0, 1 M NaCl followed by 0.1 mM Na-phosphate buffer pH 7.0. The washing with high- and low-salt solutions was repeated three times and the bound nucleoprotein complexes were eluted with 3 M NaSCN containing 10 mM Na-phosphate buffer pH 7.0 and dialysed against 0.1 mM Na-phosphate buffer pH 7.0 at room temperature for 24 h. The dialysed sample was incubated with 0.1 mg ml⁻¹ proteinase K, 50 mM Tris-HCl buffer pH 8.0, 100 mM EDTA, 0.3% SDS at 37 °C overnight, treated with phenol-chloroform and precipitated with ethanol. The purified DNA was digested with *Hae*III, blunt-ended by Klenow fragment and cloned into λ gt10 vector. b, The insert of each phage clones was labelled by PCR²⁴ with ³²P-dCTP and hybridized to a set of nitrocellulose replica filters, each representing $\sim 2 \times 10^4$ plaques of immunoenriched populations, and hybridized to restricted total mouse DNA on a Southern blot. Plaque and genomic Southern hybridizations were as described²⁵.

were used as probes for cross-species hybridization. A restriction fragment, BS0.8, located 2.0 kb from the sequence of clone 208 hybridized to single-copy restriction fragments of bovine and chicken DNAs (data not shown). As *Hox-C8* is expressed embryonically, mouse embryo cDNA libraries were screened with a radiolabelled fragment of BS0.8, identifying a clone with a 3.3 kb insert, mc28. Overlapping clones covering the entire coding sequence were isolated by screening a mouse embryo cDNA library primed by a synthetic oligonucleotide corresponding to the 5' end of mc28 (Fig. 2b). The 4,279 nucleotide sequence obtained contained an open reading frame of 3,102 nucleotides coding for a polypeptide of 1,034 amino acids with a calculated M_r of 112,310 (Fig. 2c).

The sequence of the candidate *Hox-C8* target gene was significantly homologous to that of a *Drosophila* tumour-suppressor gene, lethal (2) giant larvae (*l(2)gl*)¹¹ (Fig. 3). Because no mammalian homologue of *l(2)gl* has been reported, we have termed this gene *mgl-1* (mammalian homologue of *Drosophila* lethal (2) giant larvae). Homozygous mutations of *l(2)gl* result in neoplastic transformation of neuroblasts in imaginal discs and the presumptive adult optic centres of the larval brain¹². The

mutant phenotype and subcellular localization of *l(2)gl* indicate that it may be a cell adhesion molecule¹³, and the same may be true of *mgl-1*. The sequence of the genomic regions corresponding to mc28 and mc28-5 (Fig. 2a) shows that the gene is composed of 21 exons and spread over 15 kb of genomic DNA with the sequence corresponding to the immunopurified clone (208) in the 17th intron (Fig. 2a).

<i>mgl-1</i>	1	MMKF-RFRGGADPQREKLKQELFAHKTVEHGFNPQPSALAFDPELRIMAIGTRSGAVK
<i>l(2)gl</i>	1	MFKFIIRKGGQPSADRRRLKQDLFAFKRTAQGHGFPHKPSALAYDPVLKMAIGTGTGALK
<i>mgl-1</i>	62	IYGAPGVETGLH---RDAATVTQMFL---PQGRLLTLDDSSLHLWEIHHNGCAHL
<i>l(2)gl</i>	61	VFGQPGVELYQHTLLNNSASELNVQLLEWVYGTGRILSLTAANQLILWE-----
<i>mgl-1</i>	114	EEGLSFHPPSRPSFDNASFLTRVTVV-LLVAGNTAALGTESGIFFLDVATLALLEG
<i>l(2)gl</i>	111	PVGATLLPIKTLFPD----GKLKVVSLCCSLSKDLLWIGTEGGNIYQLDLHTFTIKE-
<i>mgl-1</i>	173	QTLSPDVLRSVPDDYRCGKALGPVESLQGHLDQPSKILIGYSRGLLVISQATQSDNV
<i>l(2)gl</i>	165	PVIYHDDVLEQVPPAYK--LNPGAIESIRQLPNSPKLLVAYNRGLCVLWDFESASVQRA
<i>mgl-1</i>	233	FL--GNQQLSLCWGRDSSIISSHSDGSYAIWSTDGSPPTLPQTVVTPYGFPPCKAI
<i>l(2)gl</i>	223	YIAPGHGQSVGLTVNFGESFTWYHADGSYATWSIDNPEPP---SNVNVYPYGDPCCKSI
<i>mgl-1</i>	291	NKILMRSCSGDHFIIISGGMPSRSGYDRHCVSVLRAETL-VTLDFTSRVIDFTVHSTQ
<i>l(2)gl</i>	280	NR-LYKGRRSNDVIVFSGGMPRSAYGDHNCVSHASDGHKVCCLDFTSKVIDFF---VT
<i>mgl-1</i>	350	PEDECDNPOALAVLLEELVVLDTQTPGWPAPYAPYAPLHSSAITCSAHVANVPSKLWA
<i>l(2)gl</i>	335	FENNRDVAEVLVVLLEELCAVDLTDPNICAIPYLVSHVASAVTCNYLASEVQSVVE
<i>mgl-1</i>	410	RIVSAGEQOSPOPASSALSWPITGG-----RNLAQEPSQR-GLLLTGHDGTVRFWDA
<i>l(2)gl</i>	395	SILRAGDEQ--DIDYSNISWPITGGTLPDNLESVEEDATKLYEILLTGHEDGSVKFWDC
<i>mgl-1</i>	462	SGVALRPLYKLSTAGLFTDCE----HADSQAQVEDDWPFRKVGCDPYSDDPRLGI
<i>l(2)gl</i>	453	TGVLKPIYNFKTSSIIFGSEDFRDDAADMSEAQVDEGEPPFRKSGCLFDPSDDPLAV
<i>mgl-1</i>	517	QKVALCKYTAQMVVAGTAGOVLVLESEVPAEAVSVANVDLLDREGFTWKGHERLNPH
<i>l(2)gl</i>	513	KKIAFCPKTGQLVGGTAGOIVIAIDFIDLPKVSLLKYSIMNLVSRDGFVKGHDQNLNR
<i>mgl-1</i>	577	TGLL----PWPA-GFOPRMLIOCLPPAAVTAVTLHAWSLVAFGTSHGFLDYQRKSP
<i>l(2)gl</i>	573	SNLLDGEAIPPTERGVSISGLVQLPPASITCMALEASGVLVSGGTAHGLVLFDFKNFP
<i>mgl-1</i>	631	VLARCTLHPNDLSAMEGLSRVSKLKKSLRQSFRRIRKSRVSGKKRTPAASSKEANAQLA
<i>l(2)gl</i>	633	VFHRCTLNPNDLTAGEQLSRKRSFKKSLRESFRKLKGR--STRT-----NOS
<i>mgl-1</i>	691	EQTCFHDLEMTVQRRIEPRASDLSGVVRCLYFADTFLRDATHHGPTMWAGTNSGSVF
<i>l(2)gl</i>	680	NOV-PTTLEAPVERQIEARCAADDGSGVMVRCCLLFAKTYVTNNVITSPTLWSATNASTVS
<i>mgl-1</i>	751	AYALEVPAA-----TAGGEKRPEQA--VEAVLGKEVQLMHRAPVVAIAVLDGRGRPL
<i>l(2)gl</i>	740	VFLHLPLPAQTAATAVPSASGNAPPHMPRISIAQLAKEIQLKHRAPVVGISIFDQAGSPV
<i>mgl-1</i>	801	PEPYEASRLDAQAPDMQGGHAYLIASEEQKFVETLPKVSAKTKFKLTAHEGCRVRKVALA
<i>l(2)gl</i>	799	-----DQLNAGENGSPPHRVLIASEEQKFVETLPKVPINKYKLTANEGARIRRIHFG
<i>mgl-1</i>	861	TF-----ASVMSDYAEITCLACTNLGD
<i>l(2)gl</i>	852	SFSCRSIPETLQSMHGCSPTKSTRSHGDEADPNISGSLAVSRGVYNNETALICLTNMGD
<i>mgl-1</i>	884	VHVSFVPLRQVHYSCIRKEDISGIAVSCVTRHGGFYLLSPSEFERFSLSARNITEPL
<i>l(2)gl</i>	912	IMVLSVPELKRQLNAAVRRINDINGVSLCFTNSGEALYMSSEIQCIGALATSRVQPT
<i>mgl-1</i>	944	CSLDISWPONATQPRLOF--SPKLSQANGTRDII--LAPESCEGSPSSAHSKHADMEPP
<i>l(2)gl</i>	972	GVVPVE--PLENEESVLEENDAEKNKYACDEVNTVEIKNPSGISICTRPAEENVGKRS
<i>mgl-1</i>	1000	EALSPVSI--DSASGDTMLDITGDDTVYEVKDFIG
<i>l(2)gl</i>	1031	VOOVNGVINSNPQANETISSIGDITVDSVRHDLN

FIG. 3 Alignment of predicted amino-acid sequence of *mgl-1* with amino-acid sequence of *Drosophila l(2)gl*¹¹. Amino-acid residues are shown in single-letter code. Double dots indicate identity and single dots indicate homologous substitutions.

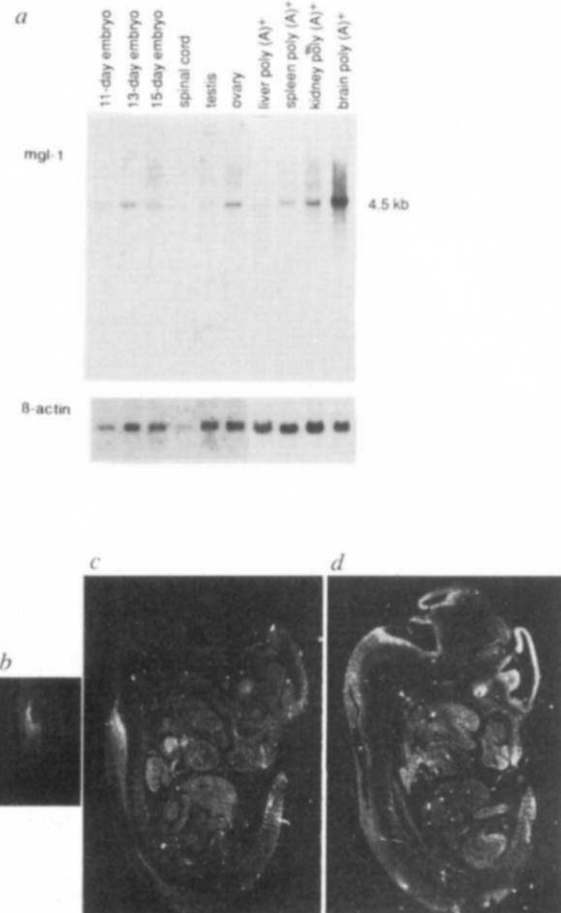


FIG. 4 Expression analysis of the *mgl-1* and *Hox-C8*. a, Northern blot analysis of *mgl-1* mRNA in mouse embryos and tissues. Total RNA of mouse embryos or adult tissues or poly(A)⁺ RNA of mouse adult tissues was hybridized with ³²P-labelled insert of the mc28. About 5 µg total RNA (11-, 13-, 15-day p.c. ICR mouse embryos, adult spinal cord, testis and ovary) or 5 µg poly(A)⁺ RNA (liver, spleen, kidney and brain) was electrophoresed. The bottom panel shows the result of subsequent hybridization with a ³²P-labelled DNA probe specific to β -actin mRNA. b-d, Immunofluorescence of Hox-C8 protein (b) and *in situ* hybridization of *Hox-C8* (c) and *mgl-1* (d) transcripts in sagittal sections of a 12.5-day p.c. embryo. Detailed analysis of *mgl-1* expression during embryogenesis will be published elsewhere.

METHODS. Total RNA from embryos and tissues²⁷ as well as poly(A)⁺ RNA²⁵ was prepared as described. Total or poly(A)⁺ RNA (5 µg) was separated in formaldehyde gel and transferred to nitrocellulose filter according to Sambrook et al.²⁵. The filter was prehybridized for 2 h at 42 °C in 50% formamide, 5 × SSC, 0.1% SDS, 5 × Denhardt's solution and 100 µg ml⁻¹ denatured salmon sperm DNA. Hybridization with ³²P-labelled DNA was under the same conditions for 14 h. The filter was washed in 2 × SSC, 0.1% SDS at room temperature for 5 min and then 0.2 × SSC, 0.1% SDS at 65 °C for 20 min three times. The washed filter for total RNA was exposed to an X-ray film five times longer than the filter for poly(A)⁺ RNA. Histological analyses were done according to published procedures^{28,29}. For *in situ* hybridization, a 0.8 kb *HindIII*-*Bam*HI fragment from a *mgl-1* cDNA clone mc28 and a 0.24 kb *SacI*-*AluI* fragment from a *Hox-C8* genomic clone mg51 were subcloned into pBluescriptII, and ³⁵S-labelled antisense RNA probes were transcribed using T7 RNA polymerase.

Northern analysis of RNAs from various adult mouse tissues and embryos using clone mc28 as a probe (Fig. 4a) showed a single major transcript of 4.5 kb in all RNA samples examined, with the highest intensity in the sample from the adult brain and the lowest in the sample from adult liver. By contrast *l(2)gl* transcripts of 6.0 and 4.5 kb were detected in *Drosophila* embryo and larvae¹⁴. To investigate how Hox-C8 may regulate *mgl-1* expression, we compared Hox-C8 and *mgl-1* expression patterns during mouse embryogenesis by immunohistochemistry and *in situ* hybridization. Low levels of *mgl-1* transcript were widespread in 12.5-day p.c. embryos, but conspicuously higher levels of expression were observed in brain and spinal cord (Fig. 4d). Hox-C8 was also expressed in spinal cord (Fig. 4b, c) but the expression of *mgl-1* and Hox-C8 was complementary, as *mgl-1* RNA was restricted to the dorsal half (Fig. 4d), whereas the Hox-C8 was largely confined to the ventral half (Fig. 4b, c). Hox-C8 may therefore downregulate *mgl-1* expression in the embryonic spinal cord. *Mgl-1* expression continues along the anteroposterior axis through the spinal cord to the telencephalon whereas the anterior boundary of the Hox-C8 expression is located within the spinal cord. Other Hox proteins may therefore regulate *mgl-1* expression in the anterior part of the spinal cord, as homeobox proteins can recognize the same DNA sequences at least *in vitro*¹⁵. Although *l(2)gl* has not been shown to be a target of the *Drosophila* homeotic selector genes, the genes involved in cell division and cell communication are potential targets of homeotic genes⁶. The possible role of *Mgl-1* in cell adhesion or tumour suppression is thus reasonable for a regulatory target of the Hox-C8 gene.

Targets for the Ubx protein have previously been immunopurified from *Drosophila* embryonic chromatin by Gould *et al.*¹⁶. Although our procedure was also based on immunopurification it differed in a number of respects. First, we fixed tissues with paraformaldehyde before the immunopurification crosslinking the proteins to each other¹⁷ and to the DNA so as to prevent dissociation of target DNA sequences from the Hox gene product and allow a thorough washing to exclude nonspecific or artificial associations between free DNA and Hox proteins. Recently, Graba *et al.*¹⁸ isolated *in vivo* targets of Ubx gene product from ultraviolet-crosslinked *Drosophila* embryonic nuclei using an analogous principle. Second, we used plaque hybridization to screen target DNA sequences, rather than *in vitro* binding of the Hox-C8 protein, because *in vivo* Hox-C8 binding sites will represent only a very small fraction of the sites available *in vitro* and it is unclear whether Hox proteins have the same DNA binding specificity *in vivo* and *in vitro*. Moreover, the larger size of the mouse genome necessitated a more stringent screening procedure than that used for Ubx targets in *Drosophila*. Finally, to detect the target gene surrounding the immunopurified DNA sequence, we used cross-species hybridization rather than looking for transcripts so as to detect genes that are expressed weakly and/or in restricted development stages or tissues.

To our knowledge, this is the first biochemical isolation of a possible target gene for a homeodomain protein in vertebrates. The overlapping expression patterns of the numerous homeobox genes, as well as complex morphological defects in Hox-targeted mice^{19, 21}, indicate that several groups of Hox-gene targets exist, and their identification will be required if the molecular mechanism of body pattern formation in vertebrates is to be understood. The present achievement should thus mark a step towards that goal. □

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Presynaptic A-current based on heteromultimeric K⁺ channels detected *in vivo*

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A WIDE variety of voltage-gated K⁺ channels are involved in the regulation of neuronal excitability and synaptic transmission. Their heterogeneity arises in part from the large number of genes encoding different K⁺ channel subunits (reviewed in ref. 1). In addition, heterologous expression studies indicate that assembly of distinct subunits into heteromultimeric channels may contribute further to K⁺ channel diversity^{2–6}. A question has been whether heteromeric K⁺ channels actually form *in vivo*, and if so, whether specific combinations of subunits could account for major K⁺ currents identified in neurons. We present here biochemical evidence that Kv1.4 and Kv1.2, two K⁺ channel subunits of the Shaker subfamily, co-assemble in rat brain. The Kv1.4/Kv1.2 heteromultimer combines features of both parent subunits, resulting in an A-type K⁺ channel⁶. Immunocytochemical evidence suggests that the heteromultimers are localized in axons and nerve terminals. We propose that Kv1.4/Kv1.2 heteromultimers may form the molecular basis of a presynaptic A-type K⁺ channel involved in the regulation of neurotransmitter release.

Using *in situ* hybridization, Kv1.4 and Kv1.2 messenger RNAs exhibit distinct but overlapping expression patterns in rat brain, with co-expression in many neurons^{7,8}. The Kv1.4 protein appears to be localized specifically to axons and terminals of neurons that express this gene⁷. Immunostaining of adjacent brain sections with gene-specific antibodies reveals that Kv1.2 immunoreactivity overlaps with Kv1.4 in several parts of the rat brain, particularly in the neuropil of the cerebral cortex, axon tracts of the corpus callosum (Fig. 1c,d), and in well defined terminal fields in the hippocampus. A striking example is the colocalization of Kv1.4 and Kv1.2 in the middle third of the dentate gyrus molecular layer and in the stratum lacunosum moleculare of CA1 (Fig. 1a,b), the layers of the hippocampal

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formation that receive the terminations of the medial perforant path projection⁹. The neurons of layer II of the medial entorhinal cortex whose axons form the medial perforant path projection express particularly high levels of both Kv1.4 and Kv1.2 mRNAs^{7,8}. Both Kv1.4 and Kv1.2 immunoreactivities are also found as large puncta in the mossy fibre tract of the hippocampus, in a pattern and distribution characteristic of mossy fibre terminals (Fig. 1e,f). These large terminals represent en passant synapses made by the axons of dentate granule cells, a cell type in which Kv1.4 and Kv1.2 mRNAs are co-expressed^{7,8}. These results indicate that Kv1.2 and Kv1.4 proteins may coexist in axons and terminals of neurons expressing both genes.

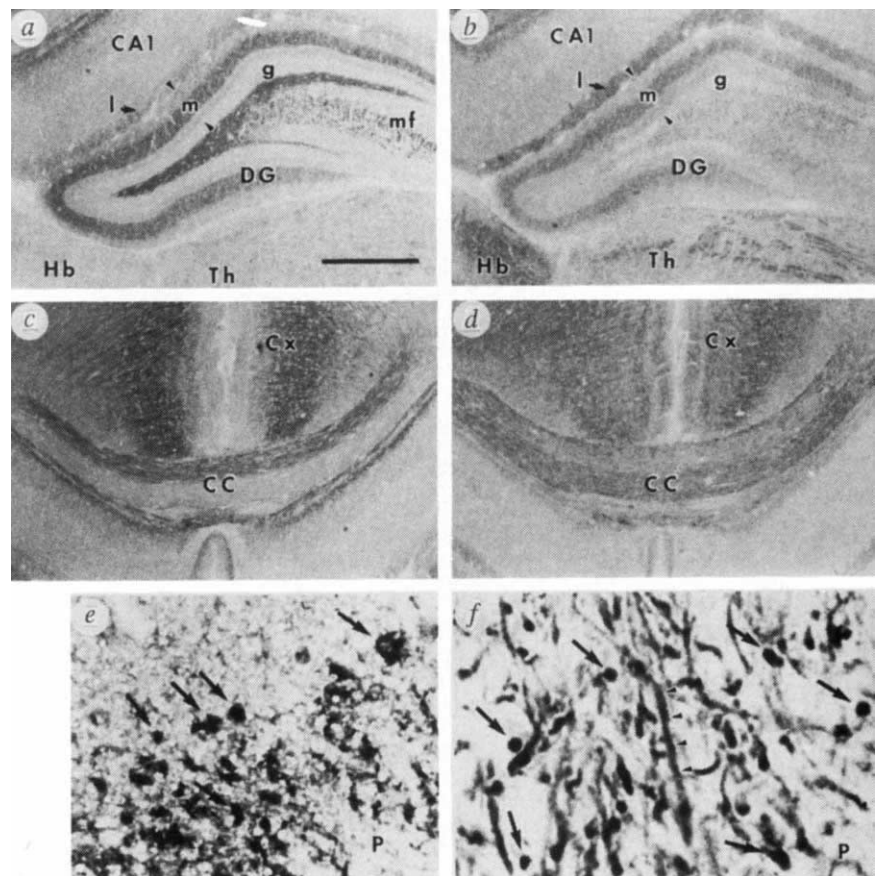
Direct association of Kv1.2 and Kv1.4 gene products in a mixed-subunit K⁺ channel could underlie their immunohistochemical co-localization. This idea is supported by biochemical co-fractionation of Kv1.4 and Kv1.2 proteins (Fig. 2). Both Kv1.4 and Kv1.2 are readily solubilized in 2% Triton X-100 and are bound by an anion-exchange (DEAE-Sephacel) column, and after elution from DEAE-Sephacel, by a wheat-germ agglutinin column. In these sequential chromatographic steps, Kv1.4 and Kv1.2 show virtually identical elution profiles with high salt and *N*-acetylglucosamine. The co-fractionation of Kv1.4 and Kv1.2 shows some specificity, because another K⁺ channel subunit, Kv4.2 (ref. 10), behaves quite distinctly on wheat-germ agglutinin chromatography, eluting over a much wider range of *N*-acetylglucosamine concentrations (Fig. 2). Interestingly, Kv4.2 (a member of the Shal subfamily) is pre-

dicted not to form heteromultimers with subunits of the Shaker subfamily¹¹, and its gene product is targeted to the dendritic domain of neurons⁷.

Copurification of Kv1.4 and Kv1.2 is consistent with protein-protein association in membranes, but could merely reflect highly similar physicochemical properties. We have used anti-peptide antibodies (raised against the non-conserved N- and C-terminal regions of the K⁺ channel subunits) to determine whether Kv1.4 and Kv1.2 polypeptides could be co-immunoprecipitated by antibodies specific for either gene. In non-denaturing conditions, Kv1.4 antibodies consistently immunoprecipitated not only Kv1.4 protein, as detected by immunoblots of the immunoprecipitate, but also Kv1.2 in a lesser amount (Fig. 3). Conversely, anti-Kv1.2 immunoprecipitates contained not only the Kv1.2 protein, but also Kv1.4. In contrast, neither Kv1.4 nor Kv1.2 antibodies immunoprecipitated Kv4.2, and antibodies against Kv4.2 failed to bring down either Kv1.4 or Kv1.2, even though they were effective in immunoprecipitating their cognate antigen Kv4.2 (Fig. 3). These findings indicate that Kv1.4 and Kv1.2 subunits associate in the neuronal membrane with some degree of specificity, perhaps reflecting properties shared by members of the same K⁺ channel gene subfamily. Identical co-immunoprecipitation results (not shown) were also obtained using samples of Kv1.4 and Kv1.2 that had been partially purified through two chromatographic steps, for example the 50 mM *N*-acetylglucosamine eluate from the wheat-germ agglutinin column (Fig. 2). The maintenance of the Kv1.4 and Kv1.2 associa-

FIG. 1 Co-localization of Kv1.4 and Kv1.2 proteins revealed by immunohistochemistry of adjacent coronal sections of rat brain. *a*, *c* and *e*, Staining pattern of Kv1.4 antibodies; *b*, *d* and *f*, staining pattern of Kv1.2 antibodies. *a* and *b*, Hippocampal region. Kv1.4 and Kv1.2 immunoreactivities overlap prominently in the middle third of the molecular layer of the dentate gyrus ('m'); the full extent of the molecular layer is delineated by arrowheads), and in the stratum lacunosum moleculare ('l') of CA1. *c* and *d*, Kv1.4 and Kv1.2 immunostaining also overlap in axon tracts in the corpus callosum (CC), and in all regions of the cerebral cortex (Cx), including the retrosplenial cortex shown here. In cortex, both Kv1.4 and Kv1.2 are found in the neuropil but not in cell bodies. *e* and *f*, Numerous large terminals in the mossy fibre tract in the CA3 region of hippocampus are immunoreactive for both Kv1.4 and Kv1.2 (examples are marked by arrows). These terminals represent en passant synapses formed by the dentate granule cell axons with the proximal dendrites of CA3 pyramidal cells (the position of the CA3 pyramidal cell body layer is indicated by 'P'). The overlap of Kv1.4 and Kv1.2 staining patterns is only partial. For instance, Kv1.2, but not Kv1.4, is present in CA3 pyramidal cell dendrites (an example is indicated by arrowheads). Additionally, whereas Kv1.2 is relatively evenly expressed throughout the thickness of the corpus callosum, Kv1.4 appears to be concentrated in the dorsal and ventral portions. Other abbreviations: DG, dentate gyrus; g, granule cell layer of dentate gyrus; Hb, habenular nucleus; Th, thalamus. Scale bars, 0.8 mm (*a*, *b*, *c*, *d*); 40 μ m (*e*, *f*).

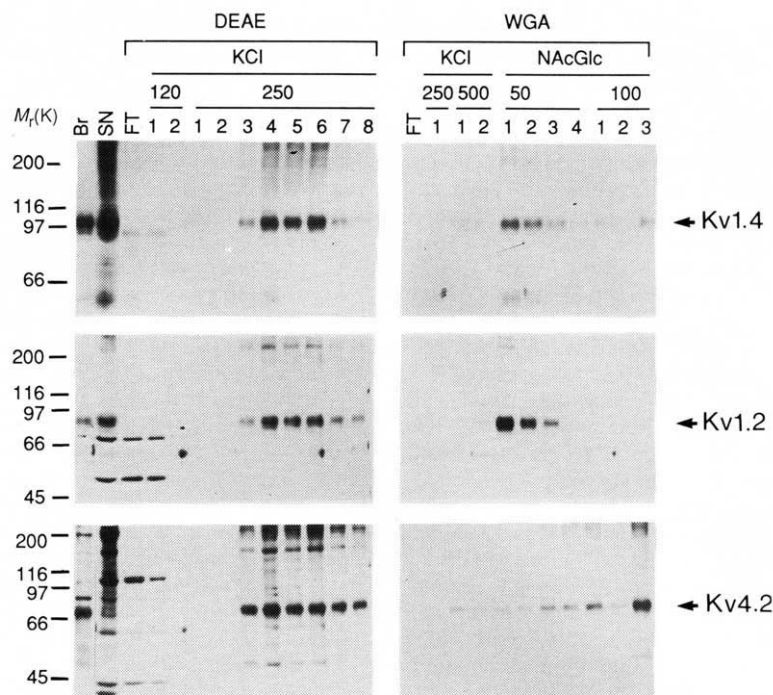
METHODS. Kv1.2 antibodies were raised in rabbits against the peptide CNEDFRENLKANTLANT (corresponding to residues 468–486 near the C terminus of the predicted Kv1.2 gene product¹²) as described⁷. Kv1.4 and Kv4.2 antibodies have been described⁷. All antibodies were affinity-purified using the specific peptide antigen coupled through the



cysteine residue to Sulfolink columns (Pierce). Immunohistochemistry was performed on sections of rat brain that had been either freshly frozen or fixed by perfusion with 4% paraformaldehyde/0.1% glutaraldehyde, using affinity-purified antibodies at 1–2 μ g ml⁻¹, and the avidin-biotin complex method⁷.

FIG. 2 Chromatographic co-fractionation of Kv1.4 and Kv1.2 subunits: Triton-solubilized rat brain membrane proteins were subjected to sequential chromatography on DEAE-Sephacel and wheat-germ agglutinin (WGA)-agarose columns. The fractionation of different K⁺ channel subunits was followed by immunoblotting with Kv1.4-, Kv1.2-, and Kv4.2-specific antibodies, as indicated. After binding of solubilized membrane proteins, the DEAE-Sephacel column was washed with 120 mM KCl; retained proteins were eluted by the application of 250 mM KCl, as indicated. Fractions positive for Kv1.4 were pooled and applied to the WGA column, which was then washed with KCl (at concentrations of 250 mM and 500 mM, as indicated). WGA-bound proteins were eluted with *N*-acetylglucosamine (NAcGlc; 50 mM and 100 mM as indicated). Numbers immediately above individual lanes refer to the fraction number collected after application of each wash or elutant. Other lanes are as follows: Br, starting rat brain membrane preparation; SN, 2% Triton extract of rat brain membranes (100,000g supernatant) containing solubilized membrane proteins; FT, flow-through fractions from the respective columns. Positions of the specific K⁺ channel polypeptides on the immunoblots are indicated, as are molecular weight standards (in thousands). Kv1.2 and Kv4.2 immunoblots were done using the same nitrocellulose filter: Kv1.2 antibodies were stripped off the blot before reprobing with Kv4.2 antibodies. Kv1.4 (*M_r* ~95K) and Kv1.2 (*M_r* ~80K) polypeptides are readily solubilized in 2% Triton and fractionate with closely similar profiles. In contrast, Kv4.2 (*M_r* ~72K) is relatively poorly solubilized in 2% Triton, and although it fractionates in part with Kv1.4 and Kv1.2 on a DEAE anion-exchange column, its interaction with WGA is different, with a substantial proportion being unable to bind WGA, and subpopulations of the rest being eluted at different concentrations of *N*-acetylglucosamine.

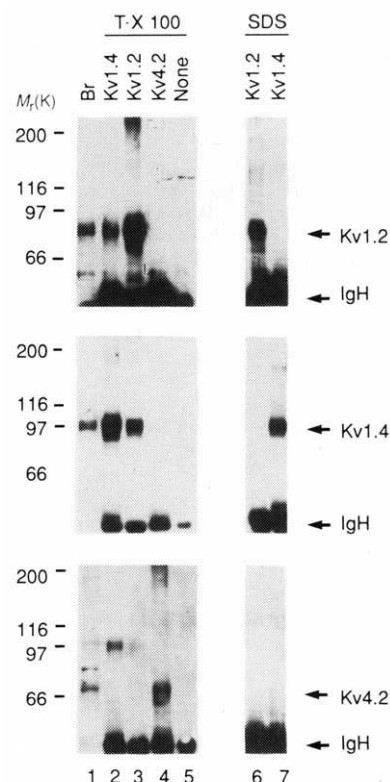
METHODS. Crude membranes⁷ prepared from 4 rat brains were solubilized for 1 h at 4 °C in 2% Triton X-100 in buffer A (20 mM HEPES, pH 7.4, 1 mM EDTA, 10% glycerol) containing 120 mM KCl. Insoluble material was pelleted at 100,000g for 1.5 h. The supernatant (~80 ml) was mixed with DEAE-Sephacel (Pharmacia; 50-ml bed volume) in batch and poured into a column. After collection of flow through, the DEAE-Sephacel column was washed with buffer A with 0.1% Triton, and with 0.02% phosphatidylcholine (buffer B) containing 120 mM KCl.



Proteins were eluted after application of buffer B containing 250 mM KCl and collected in small fractions (0.25 bed volumes). Fractions containing high levels of Kv1.4 were pooled and applied in batch to a 10-ml WGA agarose column (Vector), which was then washed sequentially with buffer B containing 250 mM KCl, 500 mM KCl, then 150 mM KCl. Proteins retained on the column were eluted with *N*-Acetylglucosamine at 50 mM, then 100 mM, in buffer B containing 150 mM KCl. Immunoblots were made⁷ with affinity-purified antibodies at 1–2 µg ml⁻¹ concentration. Filter-bound antigen was detected by donkey anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase and visualized using enhanced chemiluminescence and autoradiography (ECL, Amersham).

FIG. 3 Co-immunoprecipitation of Kv1.4 and Kv1.2 subunits. Solubilized brain membrane proteins were immunoprecipitated with affinity-purified antibodies specific for Kv1.4, Kv1.2, or Kv4.2, or mock-immunoprecipitated with no addition of antibodies ('none'), as indicated, in either non-denaturing conditions ('TX100'), or after denaturation in boiling SDS ('SDS'). Immunoprecipitates were then separated by SDS-polyacrylamide gel electrophoresis and subjected to sequential immunoblot analysis using antibodies against Kv1.2 (top), Kv1.4 (middle), or Kv4.2 (bottom). The lane marked 'Br' was loaded with 5 µg total membrane proteins from rat brain. Positions of the specific K⁺ channel polypeptides, and the IgG heavy chain derived from the immunoprecipitating rabbit antibodies ('IgH') are indicated. Immunoblots in the bottom two panels (Kv1.4 and Kv4.2) were obtained by reprobing the filter after stripping off the previous antibody; note that there is some residual Kv1.4 signal (at *M_r* ~ 95K) in the Kv4.2 immunoblot.

METHODS. Immunoprecipitations were performed from the 2% Triton X-100 extracts of whole brain membranes described in Fig. 2 (typically ~200 µg membrane protein), precleared for 1 h with protein A-Sepharose. Affinity-purified antibodies were added (3–6 µg ml⁻¹) for 2 h at 4 °C, and immune complexes collected with protein A-Sepharose. Final non-denaturing conditions (1 ml reactions) were 1% Triton X-100, 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA. For denaturing conditions, the same amount of 2% Triton extract was boiled for 3–5 min in 0.5% SDS, 50 mM Tris, pH 8, and then diluted to 1 ml (final conditions: 1% Nonidet P-40, 0.5% deoxycholate, 0.4% Triton, 0.1% SDS, 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA) before addition of antibodies.



tion through several columns makes it highly unlikely that artefactual co-immunoprecipitation occurred as a result of aggregates of membrane proteins formed during solubilization.

Immunoprecipitation of Kv1.4 by anti-Kv1.2, and of Kv1.2 by anti-Kv1.4, was abolished by boiling the membrane proteins in sodium dodecyl sulphate (SDS) before addition of antibodies. Immunoprecipitation of the respective cognate antigens, however, was relatively unaffected under these denaturing conditions (Fig. 3). These results indicate that co-immunoprecipitation of distinct subunits occurs through non-covalent interactions between Kv1.4 and Kv1.2, rather than through cross-reactivity of anti-Kv1.4 and anti-Kv1.2 antibodies. Further evidence against the cross-reactivity of the antibodies comes from the observation that, even in non-denaturing conditions, Kv1.2 antibodies fail to immunoprecipitate Kv1.4 protein from membrane extracts of GH3 cells, a cell line that expresses Kv1.4 but not the Kv1.2 gene (Lan Bo Shi and M.S., unpublished observations).

The association of Kv1.4 and Kv1.2 gene products in a multimeric complex in rat brain membranes is thus based on the following evidence: (1) co-expression of mRNAs in the same cells^{7,8}; (2) co-localization of immunoreactivities in defined structures of the brain; (3) co-purification of the proteins through two chromatographic steps; and (4) most compellingly, co-immunoprecipitation with gene-specific antibodies.

By itself, Kv1.4 encodes a rapidly inactivating K⁺ channel which recovers slowly from inactivation^{5,12}. In contrast, Kv1.2 homomultimers are very slowly inactivating K⁺ channels that are highly sensitive to dendrotoxin and 4-aminopyridine (4-AP)¹². The Kv1.4/Kv1.2 heteromultimer combines features of both of the parent subunits, forming a novel channel that exhibits fast inactivation, rapid recovery from inactivation, and high 4-AP sensitivity⁶. These characteristics are typical of A-type K⁺ channels^{13,14}, a channel type widely implicated in the modulation of the presynaptic action potential and thus neurotransmitter output¹⁵⁻¹⁸. As the pattern of co-localized Kv1.4/Kv1.2 immunoreactivity correlates with an axonal and nerve terminal location, we speculate that Kv1.4/Kv1.2 heteromultimers may underlie a class of presynaptic A-type K⁺ chan-

nels involved in regulating neurotransmitter release and synaptic efficacy.

Although we have provided evidence that Kv1.4 and Kv1.2 form heteromultimeric K⁺ channels in rat brain membranes, it should be emphasized that the overlap in distribution of Kv1.4 and Kv1.2 is only partial at both the mRNA and protein levels. Hence only a subset of Kv1.4 and Kv1.2 subunits interact with each other in the rat brain, leaving the rest to form homomultimers or to associate with other K⁺ channel subunits. Such results are to be expected if combinatorial mechanisms between different subunits contribute to the diversity of K⁺ channels in the brain. Adding another level of complexity, subunits encoded by other K⁺ channel genes may also participate in, and refine the properties of Kv1.4/Kv1.2 heteromers, thereby contributing further to the heterogeneity of A-type K⁺ channels observed *in vivo*. □

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Heteromultimeric K⁺ channels in terminal and juxtaparanodal regions of neurons

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VOLTAGE-GATED potassium (K⁺) channels display a wide variety of conductances and gating properties *in vivo*¹⁻³. This diversity can be attributed not only to the presence of many K⁺-channel gene products, but also to the possibility that different K⁺-channel subunits co-assemble to form heteromultimeric channels *in vivo*. When expressed in *Xenopus* oocytes or transfected cells, K⁺-channel polypeptides assemble to form tetramers⁴. Certain combinations of Shaker-like subunits have been shown to co-assemble, forming heteromultimeric channels with distinct properties⁵⁻⁷. It is not known, however, whether K⁺-channel polypeptides form hetero-

multimeric channels *in vivo*. Here we describe the co-localization of two Shaker-like voltage-gated K⁺-channel proteins, mKv1.1 and mKv1.2, in the juxtaparanodal regions of nodes of Ranvier in myelinated axons, and in terminal fields of basket cells in mouse cerebellum. We also show that mKv1.1 and mKv1.2 can be co-immunoprecipitated with specific antibodies that recognize only one of them. These data indicate that the two polypeptides occur in subcellular regions where rapid membrane repolarization may be important and that they form heteromultimeric channels *in vivo*.

Polyclonal antibodies specific to mKv1.1 (α -Kv1.1) and mKv1.2 (α -Kv1.2) were made by immunizing rabbits with purified fusion proteins, MBP-1.1 and MBP-1.2, each of which contains the relatively nonconserved carboxy-terminal sequences of mKv1.1 (ref. 8) and mKv1.2 (ref. 9; Fig. 1a). Another pair of fusion proteins, GST-1.1 and GST-1.2, which contain the same carboxy region of mKv1.1 and mKv1.2, were purified and used to purify the antibodies: α -Kv1.1 antibodies were affinity purified against a GST-1.1-coupled Sepharose column, and then repeatedly affinity absorbed against a GST-1.2-coupled Sepharose column which eliminated all cross reaction to mKv1.2. Conversely, α -Kv1.2 antibodies were affinity purified against GST-1.2 and immunoabsorbed against GST-1.1. These procedures yielded highly specific antibodies, as indicated by immunoblot analysis, showing that α -Kv1.1 recognizes GST-1.1 but not GST-1.2 (Fig. 1b, lanes 1, 2), and in total mouse brain protein, a polypeptide with apparent M_r 72K (Fig. 1b, lane 3). Detection of the 72K polypeptide and smaller proteins likely to be degradation products was completely blocked by preabsorp-

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tion with excess GST-1.1 fusion protein (Fig. 1b, lane 4) but not with GST-1.2 fusion proteins (Fig. 1b, lane 5), indicating that the antibody recognizes mKv1.1 protein but not the closely related mKv1.2. Similar specificity has been observed for α -Kv1.2 antibodies (Fig. 1b, lower panel). To determine whether this specificity holds for these channel polypeptides in their native conformation, we immunoprecipitated the *in vitro* translation products of mKv1.1, mKv1.2, mKv1.3 and luciferase, finding that, indeed, α -Kv1.1 and α -Kv1.2 antibodies specifically recognize mKv1.1 and mKv1.2, respectively (Fig. 1c).

Purified α -Kv1.1 and α -Kv1.2 antibodies were used to characterize the distribution of mKv1.1 and mKv1.2 immunohistochemically in mouse brain. We found that each protein was expressed widely and that, in several regions, their expression patterns overlapped. For example, each protein was expressed strongly in conical-shaped structures near the base of cerebellar Purkinje cell bodies (Fig. 2a-f), a co-localization further supported by double-labelling studies (Fig. 2g, h). Based on mor-

phology and location, these structures are likely to be the complex terminal fields of basket cells that release the inhibitory neurotransmitter γ -aminobutyric acid (GABA) onto Purkinje cells^{10,11}. Consistent with this identification, we observed that α -Kv1.1 and antibodies specific to glutamate decarboxylase (α -GAD) co-localized to these structures (data not shown). Also, α -Kv1.1 and α -Kv1.2 co-localized in some small punctate structures that often occurred in pairs along fibre tracts of the brain stem (Fig. 2i), spinal cord, corpus medullare (Fig. 2c, f, h), corpus callosum and fornix. Double-labelling experiments, however, revealed that the staining patterns were not identical. For example, the paired punctate structures were often positive to only one K⁺-channel antibody or the other (Fig. 2h, i). Differences in staining were also seen in the molecular layer of the cerebellum (Fig. 2a, c, d, f), olfactory bulb and ventral pallidum (data not shown).

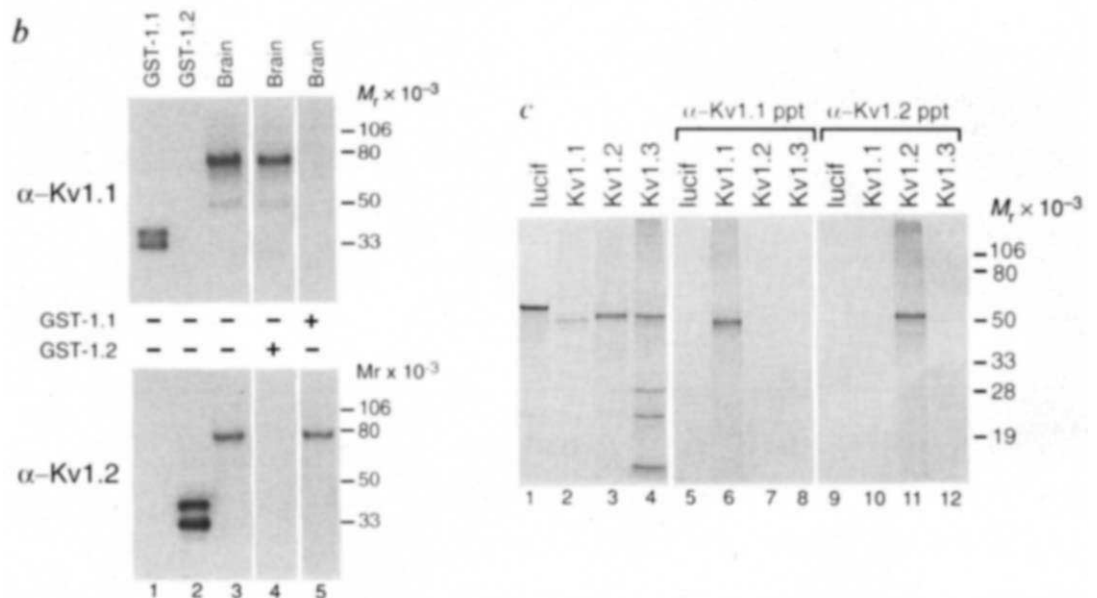
We further characterized the paired punctate staining in fibre tracts by double-labelling sections with either α -Kv1.1 or α -

a

mKv1.1 (S6)QAQ LLHV SSPNL ASDSDL SR RSSSTISKSE YMEIEED MN NSIAHYRQAN IR TGNCTT ADQN CVNKSLLTVD* (100%)
mKv1.2 (S6)--- Y-Q-T-C-KI P-SP-- KKS --A-----D ----Q-G V- --NEDF-EE- LK -A---L -NT- Y--IT-M-----* (56%)
mKv1.3 (S6)--- YM--G-CQH- S-SAEELRKA --N--L----- --V---GG-- Q-AFPQTFFK TGNS-AT--- NNNPNS---IK-IF----* (50%)

FIG. 1 α -Kv1.1 and α -Kv1.2 specifically recognize mKv1.1 and mKv1.2, respectively. a, Amino-acid sequence alignment of the carboxyl regions of mKv1.1, mKv1.2 and mKv1.3. Percentage identity with mKv1.1 is given in parentheses on the right. b, Immunoblot analysis of α -Kv1.1 (top panel) and α -Kv1.2 (bottom panel) showing specific recognition of mKv1.1 and mKv1.2, respectively. GST-1.1 (0.01 μ g), GST-1.2 (0.01 μ g) and mouse brain total proteins (40 μ g) underwent SDS-PAGE, were transferred to polyvinylidene difluoride (PVDF) membranes, then probed with each antibody alone (lanes 1-3), or each antibody preabsorbed with GST-1.2 (lane 4) or GST-1.1 (lane 5). c, α -Kv1.1 and α -Kv1.2 specifically recognize mKv1.1 and mKv1.2 in immunoprecipitation analysis. Plasmid DNAs encoding luciferase, mKv1.1, mKv1.2 and mKv1.3 were transcribed and translated *in vitro*. The resulting total translation products were subjected directly to SDS-PAGE (lanes 1-4) or were immunoprecipitated either with α -Kv1.1 (lanes 5-8) or α -Kv1.2 (lanes 9-12) before SDS-PAGE and autoradiography.

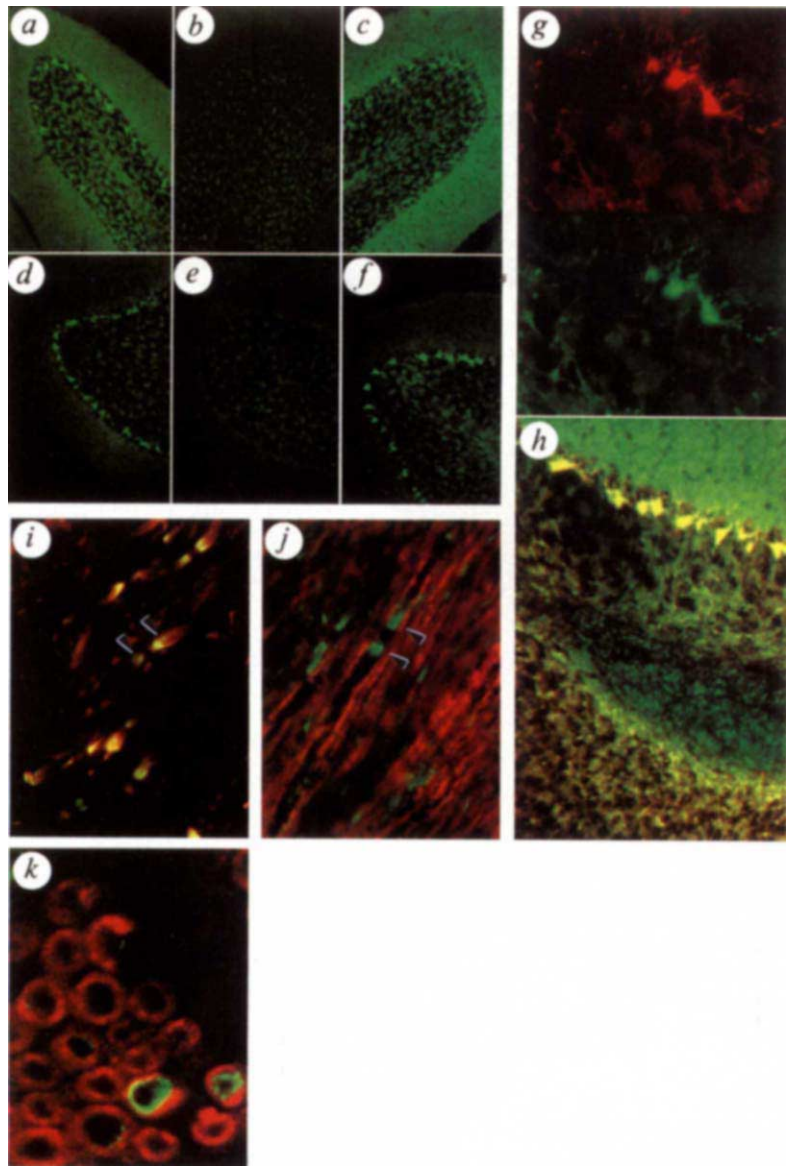
METHODS. Antibody preparation: DNA encoding the carboxy regions of mKv1.1 and mKv1.2 was cloned independently into the maltose-binding protein (MBP) expression vector, pMalC (BioLabs), generating the fusion proteins MBP-1.1 and MBP-1.2, respectively, and into a glutathione-S-transferase expression vector, pGex-3x (Pharmacia), to yield GST-1.1 and GST-1.2. All expression constructs were verified by DNA sequence analysis. The overexpressed MBP-1.1 and MBP-1.2 fusion proteins were eluted from SDS-PAGE and renatured by stepwise dialysis against 6 M, 4 M, 2 M and 0 M guanidine hydrochloride in TBS (50 mM Tris, 150 mM NaCl, pH 7.4). Each fusion protein was used to immunize rabbits as described²². GST-1.1 and GST-1.2 were affinity purified by glutathione-Sepharose CL-4B chromatography according to the manufacturer's instructions (Pharmacia) and the proteins were coupled to Affi-gel 10 (BioRad). To purify α -Kv1.1, serum was affinity purified by incubating with GST-1.1-*affi*10 resin at room temperature for 2 h, then washed with 100 column volumes of 1.0 M guanidine hydrochloride and 1.0 M



NaCl. The antibodies were eluted with 0.1 M glycine hydrochloride (pH 2.5) and immediately neutralized with Tris base powder to pH 8.0. Finally, the antibodies were immunoabsorbed repeatedly against a GST-1.2-*affi*10 column, the flowthrough fraction being collected. α -Kv1.2 was similarly affinity purified against GST-1.2-*affi*10 and immunoabsorbed against GST-1.1-*affi*10. Purified antibodies were used at 1:200 dilution in immunoblot experiments. When indicated, antibodies were preabsorbed with GST-1.1 and GST-1.2 by co-incubation (1 μ g fusion protein per μ l of antibody) at room temperature for 2 h. SDS-PAGE and immunoblotting were as described²³, except that immunoreactive proteins were visualized using the chemiluminescent Lumi-phos 530 systems (Boehringer). Immunoprecipitations: *In vitro* transcription-translation was according to manufacturer's instructions (TNT SP6 system, Promega). Expression plasmid (1 μ g) containing luciferase, mKv1.1, mKv1.2 or mKv1.3 under control of the SP6 promoter was transcribed-translated in the presence of ³⁵S-methionine. The translation products were solubilized in 1% Triton X-100 in TBS and one quarter either immunoprecipitated with α -Kv1.1 or α -Kv1.2 or subjected to SDS-PAGE analysis directly. The immunoprecipitation reactions were as described²² except 1% Triton in TBS was used as lysis and washing buffer. After SDS-PAGE, the gel was fixed in 10% acetate/10% methanol for 1 h, then autoradiographed.

FIG. 2 Localization and co-localization of mKv1.1 and mKv1.2 in mouse brain sagittal sections. *a*, Cerebellum stained with α -Kv1.1. Staining is observed in basket cell terminal fields, throughout the molecular layer, and in a punctate pattern along the fibres of the corpus medullare. *b*, Cerebellum stained with α -Kv1.1 preabsorbed with GST-1.1, which blocks specific staining. *c*, Cerebellum stained with α -Kv1.1 preabsorbed with GST-1.2. No blocking is apparent. *d*, Cerebellum stained with α -Kv1.2. Staining is again observed in basket cell terminal fields and fibres of the corpus medullare. *e, f*, Cerebellum stained with α -Kv1.2 preabsorbed with GST-1.2 (*e*) or GST-1.1 (*f*). *g*, Cerebellum double-stained with α -Kv1.1 (green) and α -Kv1.2 (red) showing especially the basket cell terminal fields. Note that each antiserum stains fine processes on the Purkinje cell soma which may be basket cell axons with boutons *en passant*. *h*, Cerebellum double-stained with α -Kv1.1 and α -Kv1.2, displayed by superimposing the images. Staining overlaps in basket cell terminal fields and in some paired punctate patterns (yellow). Differential staining (α -Kv1.1, red and α -Kv1.2, green) is seen in the central region. *i*, Brain stem double-stained with α -Kv1.1 and α -Kv1.2 displayed by superimposing the images. Note paired punctate staining (arrowheads). *j*, Brain stem double-stained with α -Kv1.1 and α -myelin basic protein (α -MyBP, red), revealing that the staining by α -Kv1.1 (green) is confined to distinct regions within the axon (arrows). *k*, Cross-section of a fibre tract double-stained with α -Kv1.1 (green) and α -MyBP (red). Note that mKv1.1 is opposed to and surrounded by the myelin sheath. *a-f*, $\times 248$; *g*, $\times 744$; *h*, $\times 496$; *i-j*, $\times 1,488$; *k*, $\times 2,480$.

METHODS. Cryostat sections of brains from young adult C57BL/6 mice were examined using purified α -Kv1.1 and α -Kv1.2. The procedures for fixation and immunocytochemistry were as described²⁴. Antibody concentrations: α -Kv1.1 and α -Kv1.2, 1:20; secondary antibodies, goat anti-rabbit IgG-FITC or IgG-Texas red and goat anti-mouse IgG-Texas red (Molecular Probes): 1:100. There was no change in the staining patterns when 0.4% dimethyl sulphoxide (DMSO) was included in the reaction or when the sections were subjected to delipidation using methanol, indicating a consistent penetration of the antibodies in the sections. Double-staining with α -Kv1.1 and α -Kv1.2 was by two separate rounds of staining. Double-staining with either α -Kv1.1 or α -Kv1.2 with α -MyBP was by simultaneous incubation with α -MyBP and α -Kv1.1 or α -Kv1.2. Images were collected on a BioRad MRC 600 scanning laser confocal microscope and printed with the Tektronix Phaser II SD Dye sublimation system.



Kv1.2 and with α -myelin basic protein (α -MyBP), which stains the myelin sheath. Results showed that both mKv1.1 and mKv1.2 might be located in or near the paranodal region of axonal nodes of Ranvier (Fig. 2*i, k*). Specific localization obtained using electron microscopic immunocytochemistry revealed that both channel proteins are highly expressed on the axon membrane of the juxtaparanodal region and in some instances in the distal portion of the paranodal region (Fig. 3*a*, data for α -Kv1.1). In the regions where the axon membrane was stained, granular staining appeared near microtubules in the axoplasm (Fig. 3*b, c*). An analogous distribution pattern has been reported for sodium channels that are concentrated in the nodal membrane, appearing also in the underlying axoplasm^{12,13}. Neither the molecular structure nor the functional role of this axoplasmic staining is known, yet it is tempting to speculate that the staining might reflect cytoskeletal associations important for the targeting or anchoring of channels at specific subcellular sites.

Given their co-localization, we sought direct biochemical evidence that mKv1.1 and mKv1.2 form heteromultimeric channels. Mouse brain membrane proteins were immunoprecipitated

with α -Kv1.2 and the ability of α -Kv1.2 to co-immunoprecipitate mKv1.1 was detected by immunoblot analysis with α -Kv1.1 (Fig. 4*a*, top panel, lane 1). Co-immunoprecipitation of mKv1.1 by α -Kv1.2 was blocked if α -Kv1.2 was preabsorbed with excess GST-1.2 protein (Fig. 4*a*, top panel, lane 2) but not when preabsorbed with GST-1.1 protein (Fig. 4*a*, top panel, lane 3), demonstrating that mKv1.1 protein is co-immunoprecipitated by α -Kv1.2 by virtue of its being assembled in mKv1.1/mKv1.2 heteromultimers, not because of a cross-reaction with α -Kv1.2. In similar experiments, α -Kv1.1 co-immunoprecipitated mKv1.2 (Fig. 4*a*, lower panel). To determine whether mKv1.1 and mKv1.2 channels might aggregate during the immunoprecipitation reaction, we varied the concentration of mouse brain proteins in the immunoprecipitation reaction over two orders of magnitude, from 0.05 $\mu\text{g } \mu\text{l}^{-1}$ to 5 $\mu\text{g } \mu\text{l}^{-1}$. At all concentrations, when >90% of mKv1.2 was precipitated (Fig. 4*b*, lane 3), about 30% of mKv1.1 was precipitated (Fig. 4*b*, lane 1). Similarly, about 30% of mKv1.2 was co-immunoprecipitated when >90% of the mKv1.1 was precipitated with α -Kv1.1 (data not shown). These data suggest that the observed co-immunoprecipitation is due to the formation of heteromultimeric channels *in vivo*.

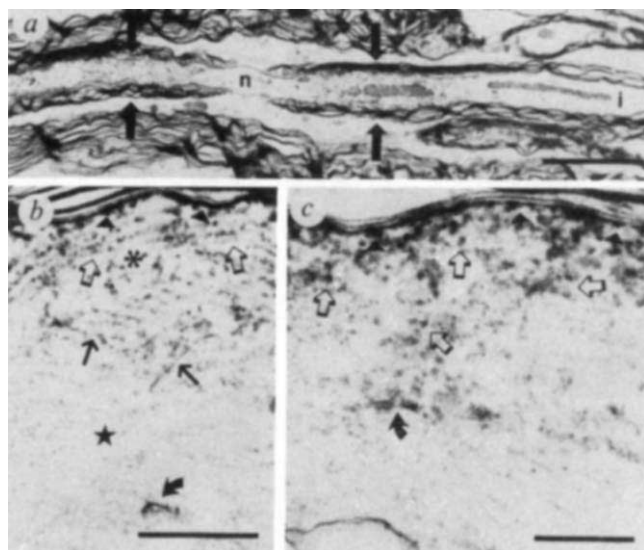


FIG. 3 Electron microscopic immunocytochemical staining of brain stem shows the expression of α -Kv1.1 in the axoplasm and on the axolemma in the juxtaparanodal regions of the node of Ranvier. *a*, In a small myelinated axon (2.5 μ m diameter) the granular immunoreaction product is seen in the juxtaparanodal regions (arrows) but not the nodal (n) or internodal (i) regions. In small axons, the immunoreaction product in the juxtaparanodal region is localized not only close to the axolemma, but also throughout the cytoplasm. Similar results were obtained using α -Kv1.2 (data not shown). *b*, *c*, Higher magnifications of large myelinated axons (4–8 μ m). The immunoreaction product appears densely associated with the axolemma (arrowheads) and within the adjacent 0.3–0.4 μ m cytoplasm (asterisk). Large immunoreactive granular densities are seen near peripheral microtubules (open arrows), giving them a darker and uneven appearance. Neurofilaments further interior (0.3–0.9 μ m) are less densely stained but have a fine granular reaction product (solid arrows). Neurofilaments in the central cytoplasm are not stained (star). Occasionally smooth endoplasmic reticulum has immunoreaction product associated with it (curved arrows). Scale bars, 4.0, 0.5, 0.25 μ m, respectively.

METHODS. Frozen sections (80 μ m) were cut on a sliding microtome. Free-floating sections were then used for immunocytochemistry using the indirect peroxidase anti-peroxidase technique of Sternberger except that we used modified dilution buffer (3% BSA + 3% normal goat serum + 0.4% DMSO + 0.05 M TBS)²⁵. The concentration of antibodies are as follows: α -Kv1.1 and α -Kv1.2, 1:40; goat anti-rabbit IgG, 1:100; rabbit PAP, 1:250. The peroxidase/DAB reaction was performed as described²⁶. Sections were processed for electron microscopy as follows. They were rinsed in 0.1 M sodium cacodylate (0.5 h), post-fixed with 1% OsO₄ in 0.15 M sodium cacodylate (1 h), rinsed, dehydrated and flat embedded in Medcast resin. Serial ultrathin sections (silver to light yellow interference colour) were cut, stained with uranyl acetate and Reynolds lead citrate and examined on a Philips 410 electron microscope.

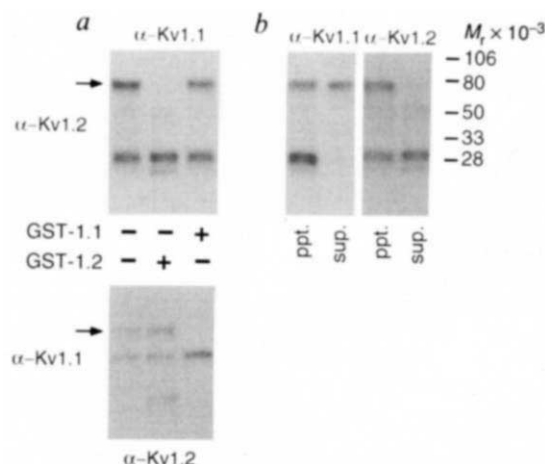
When co-expressed in *Xenopus* oocytes, mKv1.1 and mKv1.2 form heteromultimers with a rapidly activating and sustained K⁺ current, similar to that seen when either gene is expressed alone¹⁴. But significant differences are observed in the activation thresholds of each channel and in their regulation by phosphorylation¹⁵. The functional consequences of these differences at specific subcellular sites is not known. Both channel types probably contribute to the rapid repolarization of the cerebellar basket cell terminal. That precise regulation and synchronous neurotransmitter release are important at this synapse is suggested by the presence of specialized junctions, and hence presumed electrical interactions, between the various lobes of the terminal plexus¹⁰. In the juxtaparanodal regions of the nodes

of Ranvier, mKv1.1 and mKv1.2 may function to quickly repolarize the axon, thereby supporting the rapid conduction of high-frequency action potentials. Electrophysiological and pharmacological studies of demyelinated axons have reported K⁺ currents in the paranodal region with properties similar to those of mKv1.1 and mKv1.2^{16,18}.

Our finding that mKv1.1 and mKv1.2 can form heteromultimers *in vivo* confirms the principles for K⁺ channel assembly established in the *Xenopus* oocyte expression system. Previous work has shown that the assembly of Shaker-like subunits is dependent on highly conserved sequences in the cytoplasmic N-terminal region of all Shaker-like genes cloned to date, including mKv1.1 and mKv1.2^{19,20}. Furthermore, the co-assembly of more

FIG. 4 mKv1.1 and mKv1.2 can be co-immunoprecipitated from mouse brain membrane proteins. *a*, Top panel, α -Kv1.2 co-immunoprecipitates the 72K mKv1.1 protein (arrow). Mouse membrane proteins (10 μ g) were immunoprecipitated with either α -Kv1.2 alone (lane 1) or α -Kv1.2 preabsorbed with GST-1.2 (lane 2) or GST-1.1 (lane 3). The immunoprecipitated products were subjected to SDS-PAGE and immunoblotting with α -Kv1.1. Bottom panel: similar experiment showing that α -Kv1.1 co-immunoprecipitates the 72K mKv1.2 protein (arrow). *b*, Estimation of the percentage of mKv1.1 and mKv1.2 being immunoprecipitated with α -Kv1.2. Mouse brain proteins (10 μ g) were immunoprecipitated with α -Kv1.2; the immunoprecipitated products (ppt.) and half of the nonimmunoprecipitated supernatant fraction (sup.) (limited by the volume that could be loaded) were subjected to SDS-PAGE and probed with α -Kv1.1 (lanes 1, 2) or α -Kv1.2 (lanes 3, 4). The optical density of each band corresponding to either mKv1.1 or mKv1.2 was analysed with the Optimus density analysing program. Respective to each of their total proteins, we estimate that about 30% of mKv1.1 is co-precipitated when 90% of the mKv1.2 protein is precipitated using α -Kv1.2.

METHODS. Mouse brain membrane proteins were prepared as described for purification of DTX-sensitive K⁺ channel²⁷. The solubilized membrane protein fraction (in 1% Triton + TBS) was immunoprecipitated with α -Kv1.2 or α -Kv1.1. The immunoprecipitated and supernatant fractions were subjected to SDS-PAGE and immunoblotting with α -Kv1.1 or α -Kv1.2 as described for Fig. 1.



distantly related K⁺-channel genes from different subfamilies (that is, Shab, Shaw, Shal) is apparently limited²¹, perhaps because of differences in sequence between their assembly-determining domains. Thus, we predict that heteromultimer formation *in vivo* is at least partly constrained by structural barriers between channel proteins from different K⁺-channel subfamilies.

Our finding that mKv1.1 and mKv1.2 co-assemble to form heteromultimeric channels *in vivo* suggests that formation of heteromultimeric K⁺ channels may contribute to the observed physiological diversity of voltage-gated K⁺ channels *in vivo* and to the fine control of action potentials in axons and nerve terminals. □

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Inhibition of DNA replication factor RPA by p53

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THE tumour suppressor p53 specifically interferes with the onset of S phase. The mechanism of the growth suppression action of the protein is unclear, though recent evidence points to transcriptional activation and repression functions of the protein¹. A competing hypothesis suggests that p53 interacts with the DNA replication apparatus and directly interferes with DNA replication. The major evidence for this hypothesis is that p53 interacts with the simian virus 40 (SV40)-encoded protein T antigen and interferes with the ability of T antigen to unwind the SV40 origin of DNA replication, and recruit DNA polymerase α to the replication initiation complex^{2,3}. Here we report that p53 physically interacts with and inhibits the function of a cellular DNA replication factor, the single-stranded DNA-binding protein complex RPA.

RPA is composed of three polypeptides of M_r 70K, 34K and 13K, and has been evolutionarily conserved from *Saccharomyces cerevisiae* to humans^{4,5}. It is the first cellular factor that is recruited to the initiation complex and its ability to bind to single-stranded DNA is essential for the first step of DNA replication, namely unwinding of DNA at the origin of DNA replication^{6,7}. To determine whether p53 interacts with RPA, biochemically purified RPA was mixed with glutathione beads noncovalently coated with either glutathione-S-transferase (GST) or GST fused in-frame with wild-type human p53 (GST-p53). About 75 ng of RPA was specifically associated with the GST-p53 beads (Fig. 1a). For comparison, about 285 ng of T antigen was associated with the same amount of GST-p53 beads. When a crude extract from human 293 cells was mixed with GST or GST-p53 beads, about one-tenth of the input RPA was associated with the GST-p53 beads. In contrast, as the Ponceau-S stain of the blot shows, there are at least 20 major polypeptides present in the cell extract which did not associate with GST-p53 beads.

Other negative controls are described in Fig. 1 legend.

The p53-bound RPA was noticeably inhibited in its ability to bind to single-stranded DNA (Fig. 1b). Further, if RPA was pre-incubated with single-stranded DNA, and then allowed to bind to GST-p53, only non-DNA-bound RPA was found associated with GST-p53 beads. Thus p53 inhibits the ability of RPA to bind single-stranded DNA, which has interesting functional implications. This result also argues against the artefact that the observed RPA-p53 interaction is due to RPA and p53 being tethered by non-specific association with a fragment of DNA.

To determine whether RPA and p53 formed a complex *in vivo*, extracts of SaOs-2 cells (with no p53 expression), 293 cells (with wild-type p53) and CEM cells (with two mutated alleles of p53)^{8,9} were immunoprecipitated with a monoclonal antibody to p53, pAb410 (Fig. 2). About 5–10% of the total RPA present in the cell was co-immunoprecipitated with anti-p53 antibody when the lysates contained p53.

To identify which subunit of RPA interacted with p53, diploid strains of yeast expressing human RPA p34 or lexA-RPA p70 fusion derivatives were created, and the ability of the human proteins to associate with p53 monitored by immunoblotting with appropriate antibodies (Fig. 3). Up to 10% of lexA fusions containing RPA p70 or the N-terminal half of RPA p70 could associate with GST-p53 beads, whereas the lexA protein alone or the RPA p34 protein failed to associate with GST-p53. The N-terminal half of RPA p70 could bind to GST-p53 beads in the absence of p34, suggesting that this is the major p53 binding domain in the RPA holocomplex.

When T antigen was titrated into a mixture containing GST-p53 and human RPA, there was no competitive inhibition of RPA binding (data not shown). Also, when the beads were first saturated with either T antigen or with RPA, the subsequent addition of either RPA or T antigen did not show a diminution in the binding of the second protein (data not shown). This implied that T antigen and RPA bound to different parts of the p53 protein. p53 carrying a common 'hot-spot' transforming mutation R175H¹⁰, which greatly diminishes the ability of p53 to bind to T antigen, did not alter the binding of RPA (Fig. 4a). Another common transforming mutation of p53, R273H, also bound RPA (not shown). Both the transforming mutant forms of p53 also inhibited the ability of RPA to bind single-stranded DNA (not shown). Finally, different deletion derivatives of p53 fused to GST were used to map the part of p53 that was important for binding RPA (Fig. 4b). The N-terminal portion of p53,

up to the second conserved region and containing the acidic transcriptional activator region, could bind RPA, as could the C-terminal region from the fifth conserved region to the C terminus. In contrast, the middle portion of the protein from conserved region 2 to 5, which binds T antigen¹¹, could not bind RPA. The finding that two separate parts of p53 specifically

bind RPA suggests the presence in the two parts of a common structural motif recognized by RPA.

RPA wild-type p53 interaction could regulate the onset of S phase by directly inhibiting the ability of RPA to bind single-stranded DNA. But this explanation may be too simple because the common transforming mutations of p53, which do not retard

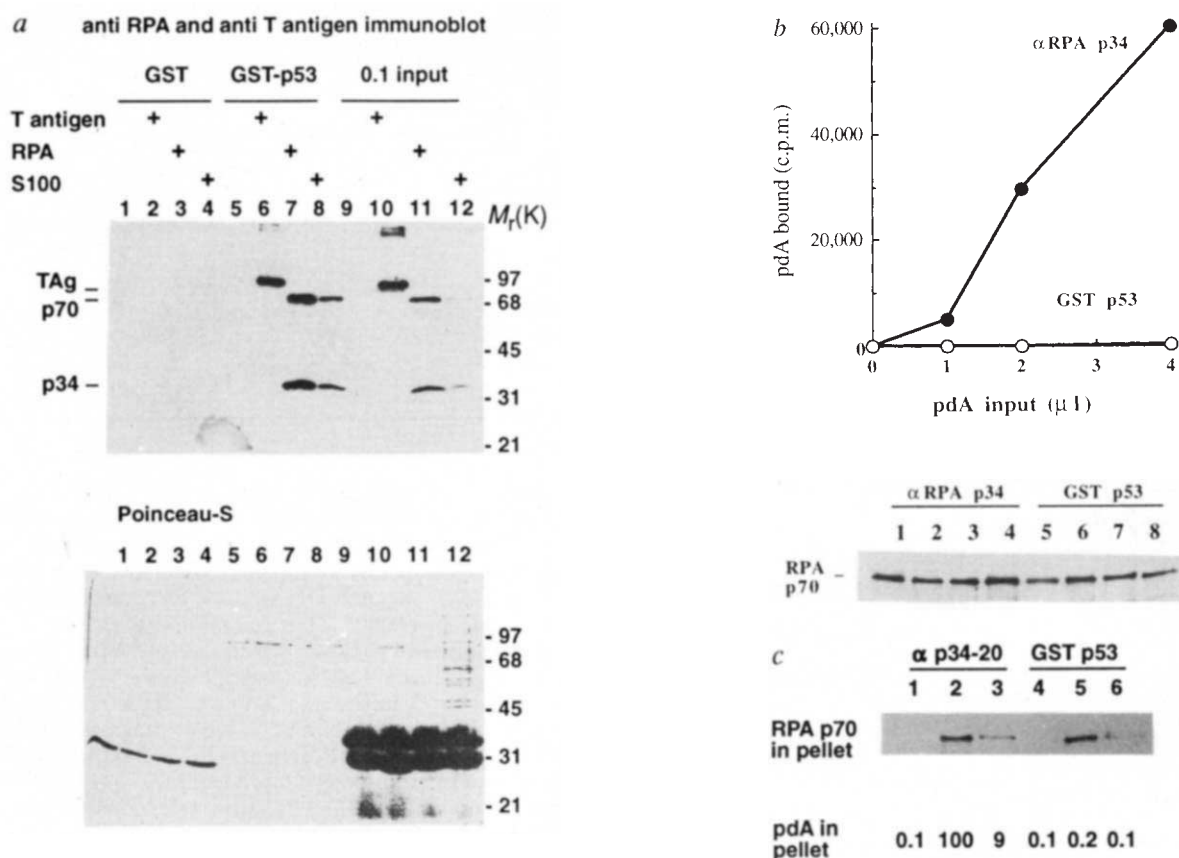
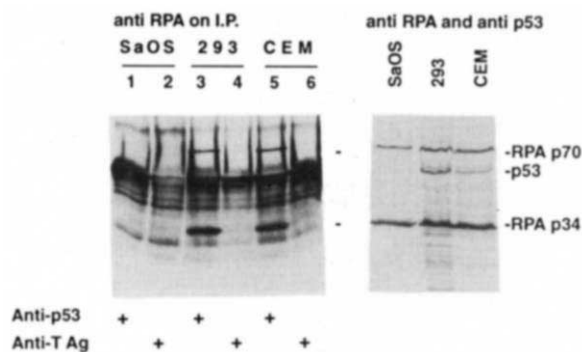


FIG. 1 Association of RPA with GST-p53 beads. **a**, Immunoblot probed with polyclonal antibody to RPA (anti-RPA) and with monoclonal antibody to T antigen (pAb419). Lanes 1–4, Proteins associated with GST beads; 5–8, proteins associated with GST-p53 beads; 9–12, one-tenth of the input proteins that were incubated with the beads in lanes 1–8. The beads were incubated with blocking protein only (lanes 1, 5 and one-tenth input in 9), with 2,850 ng SV40 T antigen (lanes 2, 6 and one-tenth input in 10), with 750 ng RPA (lanes 3, 7 and one-tenth input in 11), or with 135 μ g human 293 cell extract (lanes 4, 8 and one-tenth input in 12). The lower half shows the same blot stained with Ponceau-S. The intense 31K polypeptide is the blocking protein, casein. To control for nonspecific binding of proteins present at low concentrations in the S100 extract, ERK kinase was also followed in this experiment by immunoblotting, and was found not to associate with GST-p53 beads (not shown). GST-oct1, a fusion protein between GST and the DNA binding POU domain of the transcription factor oct1, and GST-VP16 containing the herpes simplex virus transcription activator VP16 residues 5–412 (lacking the acid activator region), also failed to bind RPA (data not shown). **b**, The amount of end-labelled single-stranded DNA (poly dA) bound by beads carrying RPA immobilized by anti-RPA antibody (anti-RPA p34 + rabbit anti-mouse IgG + protein A sepharose beads), or by GSTp53 beads, is plotted against the amount of input single stranded pdA (263,000 c.p.m. μ l⁻¹). The panel below the graph is an immunoblot with anti-RPA p70 showing that equal amounts of the DNA binding subunit were immobilized on the beads used for each of the points on the graph: RPA immobilized by anti-RPA p34 (lanes 1–4) or by GST-p53 (lanes 5–8). **c**, Different amounts of RPA (lanes 1, 4, 0 ng; lanes 2, 5, 750 ng; lanes 3, 6, 75 ng) were mixed with 18 ng radiolabelled poly dA and the RPA precipitated with anti-RPA p34 antibody (lanes 1–3) or with GST-p53 (lanes 4–6). The amount of RPA p70 present in each pellet is

shown in the immunoblot with anti-RPA p70 antibody, and the percentage of input poly dA that is co-precipitated is indicated below the lanes. **METHODS**. GST or GST-p53 were expressed in *Escherichia coli* and bound to glutathione-agarose beads as previously described¹¹. Beads carrying 400–600 ng GST and 200–300 ng GST-p53 were incubated with the indicated proteins in buffer A (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.01% NP-40, 25 mM NaCl, 1 mM DTT, 0.1 mM PMSF, 10% glycerol) containing 1% (w/v) milk protein for 1 h at 40 °C, and then washed four times with NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl pH 8.0, 0.5% NP-40). Proteins still associated with the beads were recovered by boiling in Laemmli sample buffer, separated by electrophoresis on a 15% SDS-polyacrylamide gel and transferred to nitrocellulose. The immunoblots were incubated with the appropriate antibodies and developed by the enhanced chemiluminescence method (Amersham) according to the manufacturer's instructions. **b**, The beads were washed after incubation with RPA and then re-incubated for 1 h with indicated amounts of single-stranded DNA in 100 μ l of the same buffer used for RPA binding. After washing with NETN buffer, the radioactivity associated with the beads was measured. The beads were then boiled in sample buffer, electrophoresed and immunoblotted as above. **c**, The single-stranded DNA was added to the RPA-containing buffer which was incubated with the beads as described above. The polyclonal antibody to human RPA (anti-RPA) and the monoclonal antibody to T antigen (pAb 419) have been described^{4,15}. The monoclonal antibodies to RPA p34 (p34-20) and RPA p70 (p70-9) will be described elsewhere (S. Din and B. Stillman, manuscript in preparation). RPA was purified from a 293 cell extract, and T antigen from a baculovirus expression system according to published protocols¹⁶. The 293 cell extract (S100) was prepared by hypotonic lysis of exponentially growing cells as published¹⁷.



the onset of S phase, also inhibit the function of RPA. The interaction between RPA and mutant p53 could be prevented by the mutant p53 being localized to different subcellular or subnuclear compartments relative to wild-type p53 and RPA. An alternative possibility, raised by the finding that T antigen and RPA bind to different parts of the p53 protein, is that a tripartite RPA-p53-T antigen interaction is necessary for p53 to inhibit DNA replication. In this hypothesis, the transforming

FIG. 2 Co-immunoprecipitation of RPA and p53 from cell extracts. Left, Immunoblot probed with anti-RPA antibody. Extracts from SaOs-2 cells (lanes 1, 2), 293 cells (lanes 3, 4) and CEM cells (lanes 5, 6) were immunoprecipitated with a monoclonal antibody to p53 (pAb 410) or to T antigen (pAb 419, negative control). The RPA p70 and p34 polypeptides are indicated by dashes. Right, Immunoblot of extracts from SaOs-2, 293 and CEM cells, probed with anti RPA and anti p53 (pAb 410) antibodies.

METHODS. Exponentially growing cultures of the cells were lysed in lysis buffer (50 mM Tris-HCl pH 7.4, 125 mM NaCl, 50 mM NaF, 5 mM EDTA, 1 mM Na_3VO_4 , 0.1% NP-40). The protein extracts (330 μg from SaOs-2, 660 μg from 293 and 600 μg from CEM cells) were diluted with 3 vols buffer A and immunoprecipitated with 1 μl each of ascites containing either pAb 410 (ref. 15), or pAb 419, followed by rabbit anti-mouse IgG and protein A sepharose beads using standard procedures¹⁸. The immunoprecipitates were boiled in Laemmli buffer, and processed the same way as described for the glutathione agarose beads in Fig. 1. For the right-hand panel one-tenth of the amount of each extract used in immunoprecipitation reactions was run directly on SDS-polyacrylamide gels and immunoblotted.

mutants of p53 would behave differently from wild-type p53 because though they bind RPA, they fail to bind T antigen (or the cellular T antigen equivalent). The free T antigen could then compete with p53 for RPA by virtue of the reported T antigen-RPA interaction¹² and thereby recruit RPA for replication. Future experiments will be designed to test these possibilities. Finally, the RPA-p53 interaction could contribute to the arrest of replication seen upon irradiation of cells, the attendant

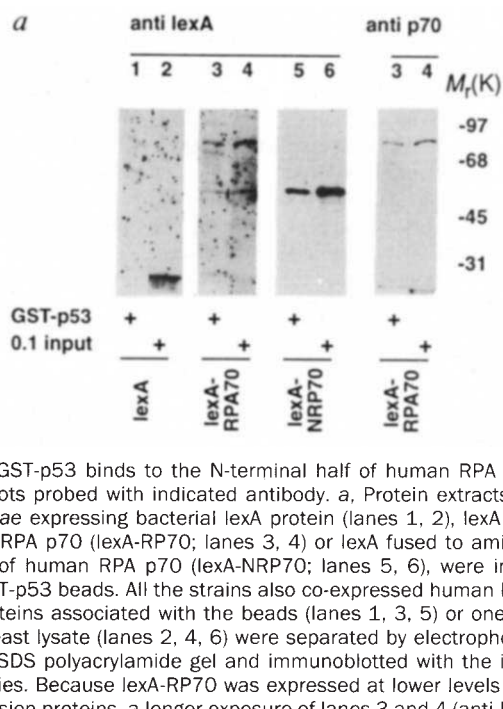


FIG. 3 GST-p53 binds to the N-terminal half of human RPA p70. Immunoblots probed with indicated antibody. a, Protein extracts from *S. cerevisiae* expressing bacterial lexA protein (lanes 1, 2), lexA fused to human RPA p70 (lexA-RP70; lanes 3, 4) or lexA fused to amino acids 1-308 of human RPA p70 (lexA-NRP70; lanes 5, 6), were incubated with GST-p53 beads. All the strains also co-expressed human RPA p34. The proteins associated with the beads (lanes 1, 3, 5) or one-tenth of input yeast lysate (lanes 2, 4, 6) were separated by electrophoresis on a 10% SDS polyacrylamide gel and immunoblotted with the indicated antibodies. Because lexA-RP70 was expressed at lower levels than the other fusion proteins, a longer exposure of lanes 3 and 4 (anti-lexA) was necessary resulting in a higher background. Hence lanes 3 and 4 were also immunoblotted with a monoclonal antibody to RPA p70 (p70-9) which recognizes an epitope in the C-terminal half of RPA p70 present in lexA-RP70 but not lexA-NRP70. b, Protein extracts from *S. cerevisiae* expressing either human RPA p34 and lexA (lanes 1, 4, 7), or only lexA-NRP70 (lanes 2, 5, 8), or RPA p34 and lexA-NRP70 (lanes 3, 6, 9). Proteins associated with GST beads (lanes 1-3), GST-p53 beads (lanes 4-6), or one-tenth of the protein extracts incubated with the beads (lanes 7-9) were separated by electrophoresis on a 15% SDS polyacrylamide gel and immunoblotted with the indicated antibodies. In the immunoblot with anti-lexA antibody, lanes containing bacterial proteins (lanes 1-6) show a background band slightly larger in size than lexA-NRP70.

METHODS. LexA or the LexA fusion derivatives were expressed under the control of an alcohol dehydrogenase gene promoter using derivatives of the plasmid EG202 (ref. 19), and molecular clone of human RPA p70 (ref. 20). Human RPA p34 was expressed under control of a Gal 10 promoter in the YEP51 plasmid^{21,22}. Extracts containing human RPA p34 were made from yeast growing in 2% galactose-containing media. Standard methods were used to make the plasmids, transform and select yeast containing the appropriate plasmids, and make the yeast protein extracts. The association with GST or GST-p53 was studied as described in Fig. 1.

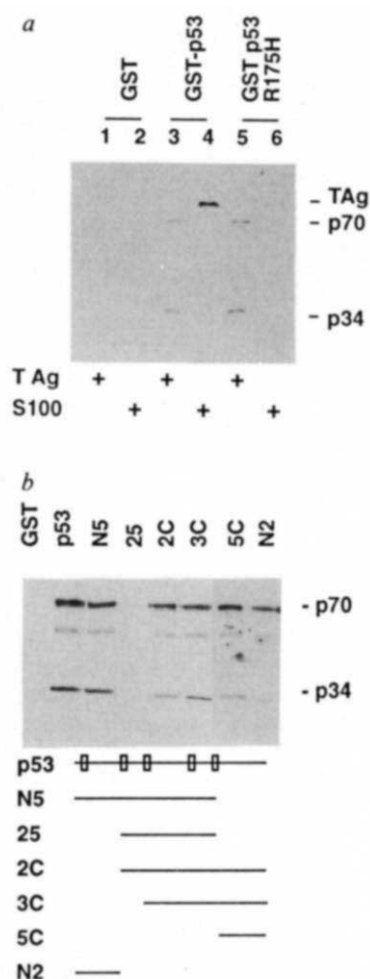


FIG. 4 Ability of mutant forms of p53 to bind RPA. Immunoblots probed with indicated antibodies. *a*, Proteins associated with GST beads (lanes 1, 2), GST-p53 beads (lanes 3, 4) or GST-R175Hp53 beads (lanes 5, 6) when incubated with 135 μ g S100 extract of 293 cells (lanes 1, 3, 5) or with 2 μ g T antigen (lanes 2, 4, 6). *b*, RPA associated with GST, GST-p53, GST-N5, GST-25, GST-2C, GST-3C, GST-5C and GST-N2 beads. The lines below the figure schematically indicate the parts of p53 contained in each of the GST derivatives. The boxes in the top line indicate conserved regions 1–5 of p53.

METHODS. The incubation and detection conditions were the same as in Fig. 1. The GST-R175Hp53 contains p53 with the common hot-spot transforming mutation at amino acid 175 (Arg to His). The other GST-fusions contain GST fused to p53 amino acids 2–293 (N5), 94–293 (25), 94–393 (2C), 155–393 (3C), 289–393 (5C) and 2–117 (N2). The construction and characterization of all except the last two derivatives is described elsewhere¹¹. The last two derivatives were constructed on the same principles using the appropriate oligonucleotides as PCR primers.

increase in p53 levels¹³ effectively preventing all the RPA in the cell from participating in DNA replication. Again, this is supported by published evidence that wild-type p53 is required for the G1 arrest of γ -ray irradiated cells¹⁴. □

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Specific commitment of different pre-mRNAs to splicing by single SR proteins

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HIGHER eukaryotic cells express a family of essential splicing factors with a characteristic RNA-binding domain and serine/arginine-rich (SR) motif¹. These SR proteins, which include SC35^{2–6} and SF2/ASF^{7–12}, are conserved from *Drosophila* to man, are required for early steps of spliceosome assembly, and can influence splice-site selections. To address their mechanisms of action, SR proteins were examined for their role in committing pre-messenger RNA to the splicing pathway. I report here that SC35 was sufficient on its own to form a committed complex with human β -globin pre-mRNA. Examination of other SR proteins and pre-mRNA substrates revealed that single SR proteins committed different pre-mRNAs to splicing with pronounced substrate specificity. These results suggest that splicing of different pre-mRNAs may require distinct sets of SR proteins, and that the commitment by SR proteins may be a critical step at which alternative and tissue-specific splicing is regulated.

Biochemically defined spliceosomal components, such as purified SR and heterogeneous nuclear ribonucleoprotein (hnRNP) proteins, instead of cell extracts^{13–17}, were preincubated with pre-mRNA to form complexes. Commitment of these complexes to splicing was determined by complementation with extracts whose ability to splice was blocked by excess amounts of competitor RNA. Although nonspecific RNAs had little effect on splicing (Fig. 1*a*, compare lanes 1 with 2 and 3), RNA containing a 5' splice site was an effective competitor for human β -globin pre-mRNA splicing (lane 4), and RNA containing a 3' splice site had a minor effect (lane 5). If the pre-mRNA was preincubated with SC35, splicing was not inhibited by any of the competitor RNAs (Fig. 1*a*, lanes 6–8), not even by the full-length human β -globin pre-mRNA (Fig. 1*b*). Thus SC35 alone was sufficient to commit human β -globin pre-mRNA to splicing. Characterization of the SC35-pre-mRNA interaction showed that SC35 binding occurred rapidly on ice and in the absence of ATP or Mg²⁺ (Fig. 1*c*), and that the pre-mRNA was committed to splicing in an SC35-concentration-dependent manner (data not shown, see Fig. 2*b*, lanes 3–6 for example). These results demonstrate that SC35 binding to pre-mRNA is necessary to initiate the splicing reaction, and that, once bound by SC35, the pre-mRNA is committed to the splicing pathway.

What is the mechanism by which SC35 commits a pre-mRNA to splicing in the presence of excess amounts of competitor

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RNA? RNA containing a 5' splice site could compete directly and specifically for SC35. But binding studies by ultraviolet cross-linking indicated purified SC35 had little RNA-binding specificity (X.-D.F., unpublished results), and previous RNase T1 protection-immunoprecipitation studies showed that SC35 became specifically associated with the 3' splice site during spliceosome assembly and that SC35 was required for the stable interaction between U1 and U2 snRNPs at the 3' splice site⁴. These observations, coupled with the current finding that SC35 binding to a pre-mRNA is sufficient to commit the pre-mRNA to splicing, strongly suggest that SC35 could change the equilibrium of splicing factors including U1 snRNP between pre-mRNA and competitor RNA. Thus, initial binding of SC35 to a pre-mRNA could be a critical and rate-limiting step for spliceosome assembly.

The assay for commitment complexes provides a new approach to studying the function of SR proteins. To this end, a number of other recombinant SR proteins including SRp20,

SF2/ASF and SRp55 were prepared identically to SC35 (Fig. 2a). In addition, several hnRNP proteins previously suggested to play roles in splicing were included to test whether any specific RNA-binding protein has a similar effect by RNA stabilization. HnRNP A1 binds to pre-mRNA with some degree of sequence specificity and affects splice site selection *in vitro*^{18,19}, and both hnRNP C^{18,20} and PTB/hnRNP I^{21,23} specifically bind to the polypyrimidine tract at the 3' splice site. The various SR proteins acted differently in committing human β -globin pre-mRNA to splicing (Fig. 2b). SC35 exhibited the highest activity followed by SF2/ASF and SRp55. SRp20 and HnRNP A1 had no detectable effect, whereas hnRNP C1 and PTB/hnRNP I inhibited splicing when pre-bound to the pre-mRNA. These results argue against the possibility that the commitment effect resulted from RNA stabilization and/or from quenching the competitor RNA by any RNA binding proteins.

The different activities of SR proteins in commitment is consistent with a recent observation that they function distinctively

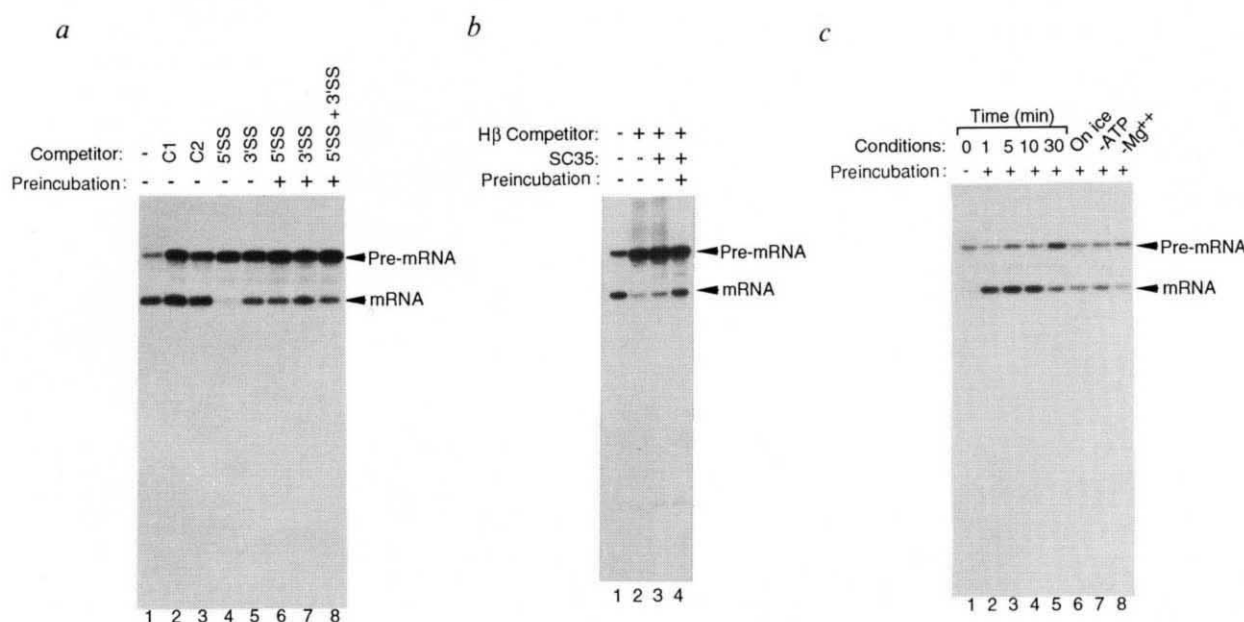


FIG. 1 Commitment of human β -globin pre-mRNA to splicing by SC35. **a**, Formation of a commitment complex with SC35. The protein concentration of SC35 was adjusted to $10 \mu\text{g ml}^{-1}$. The reactions shown contained the same amounts of SC35 ($3 \mu\text{l}$) and nuclear extract ($3 \mu\text{l}$), but differed in the order of addition and in unlabelled competitor RNAs as indicated on top. The reactions without pre-incubation (lanes 1–5) were assembled by mixing all components except labelled human β -globin pre-mRNA and unlabelled competitor RNAs, which were added together to initiate splicing. The reactions with pre-incubation (lanes 6–8) contained all components except nuclear extracts and unlabelled RNAs, which were added together after preincubation of the pre-mRNA and SC35 for 30 min at 30°C . C1 and C2 are nonspecific RNAs derived from two different plasmid sequences. 5'SS and 3'SS are the competitor RNAs containing the human β -globin 5' and 3' splice site, respectively, as previously described⁴. **b**, Use of unlabelled full-length human β -globin pre-mRNA as competitor. Splicing reactions using $3 \mu\text{l}$ nuclear extract were done in the absence of competitor RNA (lane 1) or in the presence of unlabelled full-length human β -globin pre-mRNA as competitor (lane 2–4). SC35 ($3 \mu\text{l}$) was included in the reaction without (lane 3) or with (lane 4) pre-incubation with ^{32}P -labelled human β -globin pre-mRNA substrate. **c**, Conditions required for the formation of commitment complexes. Because the unlabelled RNA containing the 3' splice site had little effect, the competitor RNA containing a 5' splice was mostly effective, and thus used in this and later experiments. Pre-incubation of pre-mRNA and SC35 was done at 30°C for different periods of time as indicated (lanes 1–5), on ice (lane 6), without ATP

and creatine-phosphate (lane 7), or without Mg^{2+} (lane 8). The omitted components were added after preincubation along with nuclear extracts and unlabelled competitor RNA.

METHODS. SC35 was expressed using a baculovirus system in Sf9 insect cells, and purified to homogeneity as described^{5,6} and modified. Briefly, 5×10^8 Sf9 cells were lysed by sonication in 10 ml buffer A (20 mM HEPES, pH 7.6; 100 mM KCl; 0.5 mM EDTA; and 1 mM DTT). After 30 min centrifugation at 35,000 r.p.m. in an SW41 rotor, the supernatant was collected, mixed with an equal volume of saturated ammonium sulphate in buffer A, and stirred for 1 h at 4°C . After 15 min centrifugation at 8,000 r.p.m. in an SS34 rotor, the supernatant was collected and loaded directly onto a 2 ml Phenyl-Sepharose column equilibrated in buffer A plus 1.7 M ammonium sulphate. SC35 was eluted from the column by step elution in buffer A plus decreasing concentrations of ammonium sulphate, and the SC35-containing fractions identified by SDS-PAGE were dialysed overnight against buffer A. SC35 was then precipitated by the addition of MgCl_2 to a final concentration of 10 mM as described⁴. The SC35-containing pellet was dissolved in buffer A and dialysed overnight against buffer A plus 10% glycerol. Before use, the protein concentration was adjusted to $10 \mu\text{g ml}^{-1}$ based on comparison with BSA visualized by Coomassie blue staining. The splicing reactions were done under standard conditions for 2 h in a total volume of 25 μl containing 0.05 pmol labelled pre-mRNA (10^5 c.p.m.), 3 μl nuclear extracts, and 3 μl SC35. Two pmol unlabelled RNAs (40-fold excess) were used as competitors to inhibit the splicing activity of nuclear extracts.

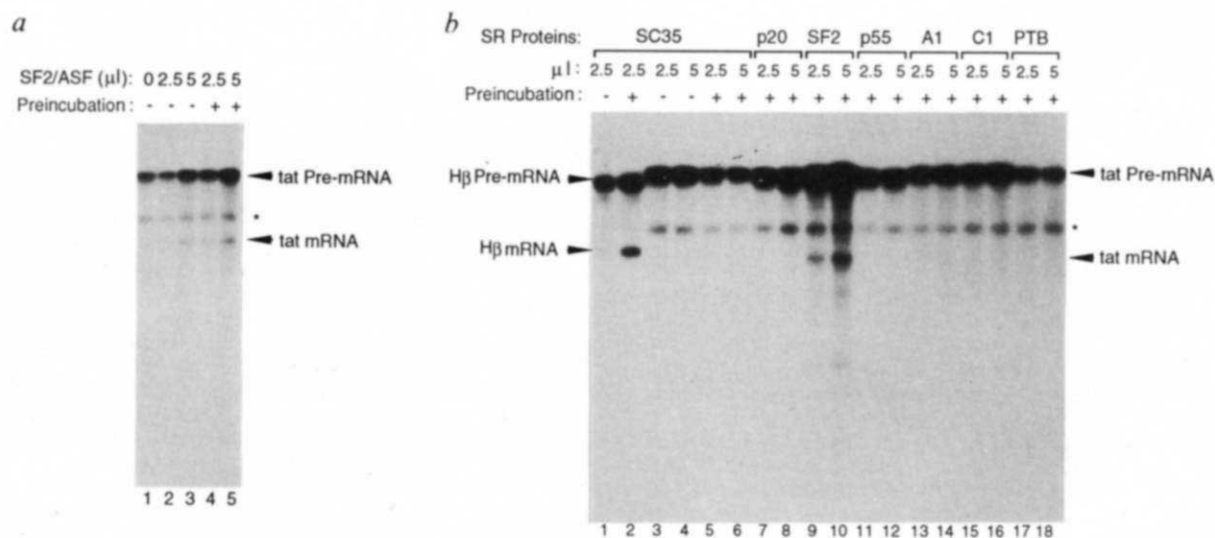
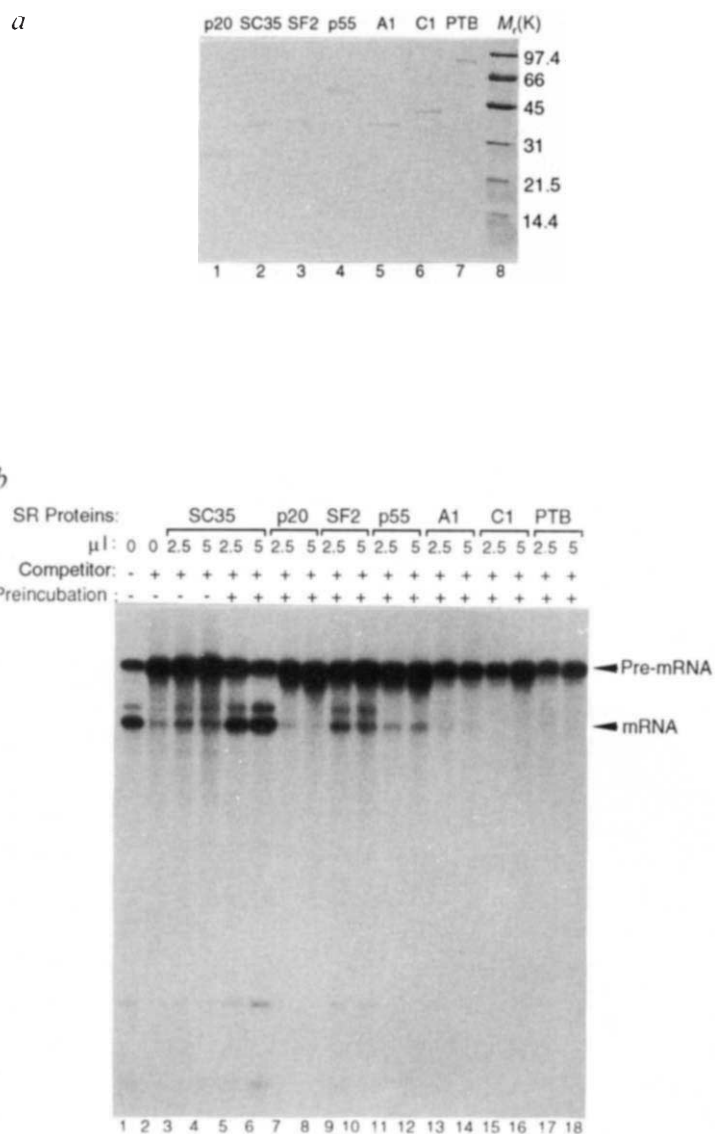


FIG. 3 Specific commitment of HIV *tat* pre-mRNA to splicing by SF2/ASF. **a**, SF2/ASF commitment of HIV *tat* pre-mRNA. The commitment assay was done in the same way as in Fig. 2b, using HIV *tat* pre-mRNA and the complementing extract preincubated with 40-fold (2 pmol) competitor RNA containing a globin 5' splice site. **b**, Comparison of SR

and hnRNP proteins in the commitment of HIV *tat* pre-mRNA (lanes 3–18). Lanes 1 and 2 are controls using human β -globin pre-mRNA and SC35. The positions of the globin and *tat* pre-mRNA and mRNA are indicated. The band indicated by the star is the result of an artefactual cleavage of the *tat* pre-mRNA (see ref. 7).

in alternative splicing²⁴, suggesting that these proteins are not functionally redundant, and that they may have overlapping, but not identical, substrate specificity. To test this hypothesis, different pre-mRNAs were tested using the same preparations of SR proteins. Figure 3 shows the results using a human immunodeficiency virus (HIV) *tat* pre-mRNA. It was previously reported that *tat* pre-mRNA splicing could be enhanced by SF2/ASF⁷. SF2/ASF also appeared to function in the commitment (Fig. 3a, compare lanes 2 with 4, and 3 with 5). Because *tat* pre-mRNA is a natural substrate for inefficient splicing, and controls could be at both early and later stages of the splicing reaction, the commitment effect was thus represented by the accumulation of both precursor and spliced products. Only SF2/ASF was able to commit this pre-mRNA to splicing, whereas SC35 and other SR proteins had no effect (Fig. 3b), in contrast to the results using the globin substrate. Again hnRNP proteins had no effect. These results clearly demonstrate that different SR proteins have different substrate specificities. In addition, the observation that SF2/ASF is required for *tat* pre-mRNA splicing indicates that the level of this splicing factor in cells may be critical to the replication of HIV.

How SR proteins achieve substrate specificity is unknown. Different SR proteins may preferentially bind to different sets of pre-mRNA, although the specificity for individual SR proteins remains to be established. Alternatively, different SR proteins may be able to recruit different spliceosomal components, so that splicing of different pre-mRNAs is dependent on one or a combination of SR proteins. A series of domain swap experiments are being done to address these possibilities.

In summary, the results of using the commitment assay to study the function of SR proteins revealed that a single SR protein is sufficient to commit a pre-mRNA to splicing, and demonstrated that different SR proteins have distinct substrate specificities. The ability of SR proteins actively to recruit previously bound spliceosomal components has general implications in the control of constitutive and regulated pre-mRNA splicing. Initial interaction of an SR protein may be critical in determining whether a given pre-mRNA is to be spliced. Similarly, an alternative splice site might be selected by an SR protein made available either by its elevated level of expression, or by the recruitment of the SR protein through specific alternative splicing regulators²⁵. □

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A move ahead on headache

The genetics of inherited migraines is proving more tractable than might have been expected given the clinical heterogeneity of the malady.

It is surely a sign of the times that clinical geneticists are willing to take on such ephemeral conditions as headaches and try and pin them down genetically. Who in their right mind would choose to tackle a condition that for a long time has defied definition, not to mention diagnosis or genetic analysis? Yet this is precisely what a French group, led by Elisabeth Tournier-Lasserre (Enfants Malades, Paris), has done and with some success. In the September issue of *Nature Genetics*, Joutel *et al.*¹ describe a linkage study that shows that a particular type of migraine maps to a 30-centimorgan region of chromosome 19.

With any other condition such preliminary linkage would only rarely warrant publication before the region of interest had been narrowed down a little further, but one look at what is involved in the genetics of headaches is enough to see the major advance that this represents. It is also enough to appreciate that the French group is not dealing with the morning-after symptoms of a good night out, but rather with a specific and very rare type of inherited migraine which has a reasonably clear autosomal dominant mode of inheritance and some very serious symptoms other than a sore head.

In general, migraine is a common and distressing type of headache for which there is no known aetiology. It affects more women (estimated at 18 per cent) than men (6 per cent) and shows broad clinical heterogeneity. In the absence of laboratory-based diagnostic tests it has, until recently, been difficult to establish whether two neurologists might be experiencing the same headache, never mind what is going on inside the head of, for example, a five-year-old victim of migraine. To address this problem of diagnostic variability, the International Headache Society proposed a new classification system that would allow all types of headache, including migraine, to be defined using a common set of rules.

Also in this month's *Nature Genetics*: two reports show Charcot-Marie-Tooth neuropathy type 1B is associated with mutations in the myelin protein *Po*; hemizygosity of the elastin gene causes Williams syndrome; point mutations in *c-myc* are frequent in Burkitt's lymphoma and mouse plasmacytomas; and *IGF2* is imprinted in mouse but not in humans.

Migraine is thus generally defined as a severe and pulsating head pain that lasts for several hours and is associated with nausea and perhaps vomiting. Approximately ten per cent of migraines are preceded by what neurologists refer to as an aura, and it is into this subgroup that familial hemiplegic migraine — the subject of the new paper by Joutel and colleagues — falls.

In familial hemiplegic migraine (FHM), the aura consists of hemiplegia (complete paralysis of half of the body) or hemiparesis (partial paralysis). This motor disturbance phase, which can be brought on by trauma to the head, typically lasts for about an hour. Although there can be some delay between the end of the aura and the start of the headache, the severe migraine-type headache may follow immediately.

For some forty or fifty years it has been clear that genetic factors are at play in migraine. In light of the efficiency with which linkage studies can be carried out these days, it is perhaps a little surprising that no one had considered collecting specimens from affected families before with a view to linkage analysis. But a look at the complexity of headache diagnosis explains this reluctance. In fact, the current discovery came about in part through a fortunate coincidence.

During investigations of a much more severe and what has, until now, been considered a completely separate disorder, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), it was noticed that some of these patients also suffered recurrent attacks of severe headache. With serendipity in full flight, it was postulated that, although clinically distinct, the two disorders might be allelic.

CADASIL was previously mapped to chromosome 19 (by the same group²) and thus close markers were available. Tournier-Lasserre and colleagues chose two well defined FHM families (made up of more than fifty individuals, thirty of whom were affected according to the criteria of the International Headache Society) and performed linkage analysis using the CADASIL-linked markers. To everyone's surprise and delight, they turned up a combined positive lod score of over 8 in favour of linkage to chromosome 19. Multipoint analysis suggested that both CADASIL and FHM mapped to a 30-centimorgan region, and although

preliminary ordering of some half-dozen markers and the two disease loci suggests that the two conditions most probably map to different intervals, it is still possible that they could be due to defects in a common gene. Any suggestion that CADASIL and FHM are associated comes as a surprise to neurologists, who have always considered the two conditions to be totally independent of one another. This work has therefore forced a rethink of the possible aetiology of both CADASIL and inherited migraine. More families are being recruited into the study, which will now concentrate on narrowing the chromosome-19 region of interest and defining the nature of the link between CADASIL and FHM.

The French group reported their exciting news at the sixth congress of the International Headache Society, held in Paris this summer. The congress organizer, Marie-Germaine Bousser, a prominent figure in the society (and a senior author on the Joutel *et al.* paper) points out that previous meetings have been dominated by discussion of drug-based treatments for migraine. For the first time, the congress devoted a full session to the genetics of migraine, with six independent groups presenting results of family-based studies. This reflects a growing awareness that many types of severe headache may show multifactorial inheritance. Tournier-Lasserre and her colleagues are therefore interested in examining other types of migraine headache that have similar although distinct characteristics for chromosome-19 associations. Other categories of headache, such as the very common and clinically even more diverse tension-type headaches, are thought to be caused mainly by environmental factors and to have little or no familial association.

As for help for those of us who suffer what we consider to be migraine, but which is in truth only a very trivial and temporary headache, we can take comfort in the knowledge that we have available to us the same treatment that is relied upon by the less fortunate sufferers of FHM: an aspirin.

Adrian J. Ivinson

Adrian J. Ivinson is Assistant Editor of Nature Genetics.

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Non-injection methods for the production of embryonic stem cell-embryo chimaeras

Stephen A. Wood, Nick D. Allen, Janet Rossant, Anna Auerbach and Andras Nagy

The simple aggregation of pluripotent ES cells with morulae-stage embryos can result in viable chimaeras at a similar frequency to that of blastocyst injection. The savings in time and equipment inherent in the aggregation techniques, however, make this approach well worth considering.

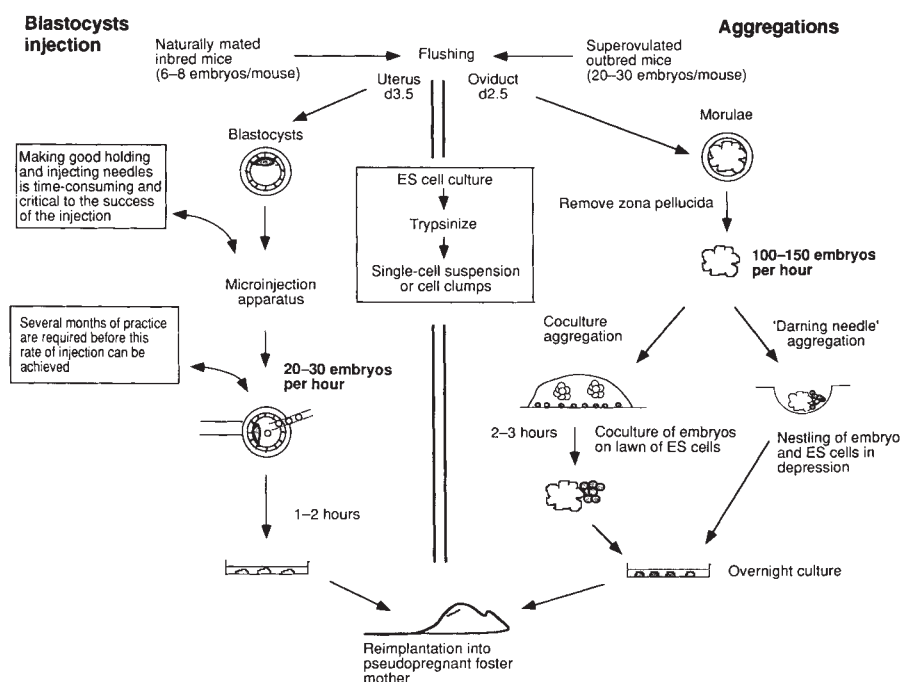
THAT murine embryonic stem (ES) cells can be used as a bridge between genetic manipulations *in vitro* and biological analysis *in vivo* is one of the most significant findings of the last decade. This unique property is made possible because ES cells can be maintained as a stable diploid population *in vitro* and upon their reintroduction into host embryos, they can also colonize all fetal tissues, including the germline. Furthermore, ES cells can be stably transfected and subsequently offer a route to transgenesis following extensive *in vitro* screening¹. This has been repeatedly demonstrated by the homologous recombination and gene-trap approaches in which rare genetic events need to be identified before analysis *in vivo*².

The production of ES cell-embryo chimaeras is a pivotal point in these experiments. Currently methods for their production fall into two groups; (1) micro-injection and (2) aggregation. The predominant method used so far has been the injection of 10–15 ES cells into the blastocoel cavity followed by transfer into pseudopregnant recipients³ (see figure). While this method is effectively used to produce chimaeras, including germline chimaeras, it does have a number of practical limitations. The main one being the cost both in equipment and man-hours. The manipulation apparatus is necessarily elaborate and correspondingly expensive. Equally as important are the number of hours, or more correctly weeks or months, required for one to acquire the necessary dexterity to introduce successfully the ES cells into the blastocoel. These considerations have lead to the investigation of alternative methods for chimaera production.

Recently, two simplified techniques have been developed that require neither expensive equipment nor high levels of expertise^{4–6}. Although differing slightly in detail, they are both based on the observation that ES cells, like embryonic carcinoma cells^{7–9}, readily aggregate with morulae and therefore all that is required for chimaera production is to bring the two cell populations into contact. The chimaeras are then cultured overnight to the blastocyst stage before transfer into pseudopregnant recipients (see figure). Although the ES cells initially attach to

the outside of the embryo they are very efficiently internalized such that by the blastocyst stage nearly all (96 per cent) chimaeric embryos contain large numbers of ES cell-derived cells in the inner cell mass from which the embryo proper develops⁶. Although these coculture experiments demon-

strate that viable normal mice can be produced and also transmit ES cell-derived material through the germline, the primary focus of analysis was on mid-gestation embryos. A large body of data has since accumulated, however, which facilitates the direct comparison of the morulae



Alternate routes for the production of ES cell-embryo chimaeras. The maintenance and preparation of ES cells are very similar for either route. The main differences arise, however, from the time needed to (1) learn the necessary manipulative skills and (2) the speed at which chimaeras can be produced once proficient in the technique. Although both methods require the ability to isolate and pipette preimplantation stage mouse embryos for aggregation techniques, this is all that is required. Blastocyst injection needs, in addition, the careful production of holding and injection pipettes, the quality of which is a major factor in determining injection success. The skills of manoeuvring embryos and cells on a microscope stage and the ability to penetrate the zona pellucida and trophectoderm while leaving intact chimaeras requires additional weeks of practice. After several months, the successful injection of 20–30 blastocysts per hour can be attained. The main advantage of aggregation techniques is the large number of embryos that can be handled and the relatively short time needed to acquire the skills to be able to do so. The 'darning needle' approach involves the manipulation of individual embryos but once placed in the darning needle depression nothing further is required until reimplantation. Coculture handles embryos *en masse* but these must be transferred from the lawn of ES cells into fresh culture drops without the carryover of too many ES cells. Full details of the coculture techniques are presented in ref. 6. The darning needle technique is essentially the same as detailed by Nagy and Rossant in ref. 5, except that ES cells are aggregated with single morulae and not sandwiched between two.

Comparison of germline chimaera production by 'darning needle' aggregation of morulae and blastocyst injection

Technique	Embryos transferred	Newborns	Chimaeras		Germline transmitters ^(a)
			Males	Females	
Morulae aggregation	321	89 (28%)	31 (9.6%)	1 (0.3%)	11 (3.4%) ^(b)
Blastocyst injection	631	250 (40%)	72 (11.4%)	23 (3.6%)	24 (3.8%) ^(c)

Donor ES cells were clones derived from the R1 line after *in vitro* selection for homologously recombined loci. A total of 10 independent lines were tested (5 by each technique). Note: percentages are based on the number of embryos transferred. (a) All highly chimaeric and a few moderately chimaeric males were tested. (b) 10 of 11 were 100% germline transmitters. (c) 15 of 24 were 100% germline transmitters.

aggregation and blastocyst injection techniques for the production of germline chimaeras, which, for many purposes, is the ultimate test for any alternative technique.

Aggregation versus injection

A series of experiments were performed, using the 'darning needle' aggregation technique, in which clones from the R1 ES cell line¹⁰ were used to form chimaeras. Each clone was selected *in vitro* for the presence of a homologously recombined locus. Chimaera production was either by injection of 10–15 cells into inbred C57BL/6 blastocysts or aggregation of a similar number of cells with a single random-bred CD-1 morula. The results from these experiments are presented in the Table. The most striking finding was that the percentage of germline chimaeras was the same for both methods. As one would expect from a male ES cell line with good developmental potential almost all chimaeras produced by morulae aggregation were males and of the germline transmitters nearly all passed on the transgene to 100 per cent of their offspring. This supports previous observations that the introduction of ES cells into earlier stage embryos results in an almost 'all-or-none' phenomenon^{4,6,10–12}.

Another advantage of morulae aggregation is the significantly reduced 'hands-on' time required as manipulative steps are kept to a minimum (see figure). The task of getting the ES cells into the centre of the embryo is efficiently undertaken by the two cell populations and requires no interference beyond the initial contact. In practice, therefore, the decreased involvement of the experimenter can mean that more chimaeras can be produced in a given time. For approaches such as gene trap^{13,14}, this can significantly increase the number of clones screened *in vivo*.

Critical parameters

A number of factors affecting both embryos and ES cells are, however, crucial for the successful production of viable chimaeras and need to be rigorously maintained for optimal results.

ES cells. The pluripotency of the ES cells is the most critical parameter in the successful production of chimaeras following aggrega-

tion with morulae. Although the precise requirements for maintaining pluripotency are not known the rigorous application of a number of principles in culturing ES cells³ as well as using early passage cells¹⁰ significantly increases the chances of obtaining highly chimaeric mice. In addition, the recent isolation of the 100 per cent pluripotent R1 ES cell line represents another significant advance¹⁰. The R1 cell line was isolated by its ability to produce viable mice after aggregation with tetraploid embryos which is an extremely vigorous test of ES cell pluripotency. Tetraploid cells are effectively selected against in the embryo but develop well in the extra-embryonic membranes. This results in the production of 100 per cent cell culture-derived mice. A number of cell lines can produce totally ES cell-derived fetuses and even newborns but these die at or shortly after birth⁴. Mice generated with the R1 line, however, are viable, normal and fertile¹⁰. Although in these experiments ES cell clumps were sandwiched between two tetraploid morula, the 'darning needle' aggregations of R1 cells with single normal diploid embryos also results in efficient germline chimaera production (see table). A number of other ES cell lines including CCE⁶, D3 (S. A. W., unpublished observations) and three parthenogenetic ES cell lines (N. D. A., unpublished observations) have also given rise to germline chimaeras after coculture at frequencies similar to those obtained with blastocyst injection.

As a consequence of the pluripotency of the ES cell used, the number of cells in each aggregate will also influence the generation of viable chimaeras. This arises from the fact that the earlier introduction of the ES cells results in higher levels of chimaerism whether by coculture/aggregation^{4,6,10} or injection^{11,12}. Although this may increase the chances of colonizing the germline¹², it also highlights any inadequacies in the ES cells used. This often manifests itself as abnormal development and resorption of mid-gestation embryos^{4,6,11}. One way to counterbalance these effects is to limit the number of ES cells that attach to the embryo by either regulating the cell density in coculture⁶ or aggregating ES cell clumps of a specific size¹⁰. In both instances the association of between 5 to 10 ES cells can result in the normal development of highly chimaeric mice.

Host embryos. Several combinations of ES cell lines and morulae from differing mouse strains have now resulted in viable chima-

ras containing significant amounts of ES cell-derived material^{4,6,10}. A clear advantage of this approach has been the use of random bred or hybrid strains of mice as they superovulate more readily, in comparison to inbred strains. Although random bred mice have given rise to germline chimaeras following blastocyst injection they can be obtained at higher frequencies using inbred strains¹⁵.

Aggregation with both precompacted and compacted embryos produces normal chimaeras. For technical reasons, however, morulae undergoing or having just completed compaction are used as the ES cells adhere more strongly to these embryos and precompacted embryos lacking the zona pellucida may break into individual blastomeres, especially if excess ES cells need to be removed by pipetting.

The media used in both techniques described so far overwhelmingly favours the growth of the embryo thereby impairing the expansion of the ES cells. In both cases, pilot experiments indicated that the use of conditions tailored for ES cells resulted in overgrowth of the chimaera by the ES cells (S. A. W., N. D. A. and A. N., unpublished observations). Under these conditions chimaeras, which had implanted, were completely, or almost so, ES cell-derived but had severely abnormal phenotypes. One possible explanation is that the extra-embryonic membranes fail to develop properly as there is evidence that the pluripotency of ES cells is limited in these lineages^{11,16}.

Conclusions and prospects

The production of viable, highly chimaeric mice can be achieved by the simple aggregation of ES cells with morulae. With the use of early passage, pluripotent ES cell lines, such as R1, the efficiency of chimaera production, including germline chimaeras, is equal to that of blastocyst injection in nearly all of the aspects analysed so far. Two aspects in which aggregation techniques have a clear advantage, however, are the significantly reduced investments in equipment and time required gaining the necessary expertise. In terms of equipment, nothing more elaborate than a Pasteur pipette is needed and the elementary skills of preimplantation embryo manipulation are all the expertise required. These are significant practical considerations and are especially relevant to anyone about to embark on chimaera production for the first time. The production of chimaeras by non-injection techniques, therefore is a very appealing alternative and should make experiments involving the introduction of genetic alterations into the germline of mice increasingly accessible to a wider range of scientists. □

We thank Wendy Pascoe, Jens Hirchenhain and Louise Aldridge for discussion of this

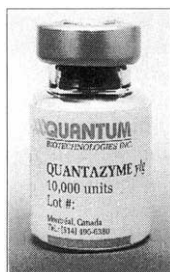
work and to the postdoctoral researchers and students who produced genetically altered ES cell lines for this comparison. Stephen A. Wood is in the Department of Molecular Embryology, Max-Planck Institut für Immunbiologie, Freiburg, Germany. Nick D. Allen is at the Agricultural and Food Research Council, Babraham Institute, Cambridge, United Kingdom. Janet Rossant, Anna Auerbach and Andras Nagy are at the Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada. For more information, fill in reader service number 100.

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Innovations in immunology

Cell culture systems for the growth of endothelial cells and keratinocytes, a reagent for the removal of free label from antibody-label conjugate preparations and a radiochromatography flow monitor are featured this week.

QUANTAZYME γ lg from Cellon Sarl is a highly active, recombinant yeast lytic enzyme (β -1,3 glucanase) that is intended for use in all processes that require digestion of the yeast cell wall (*Reader Service No. 101*). The company says that as the reagent is stable, free of detectable nucleic acids,



Yeast lytic enzyme.

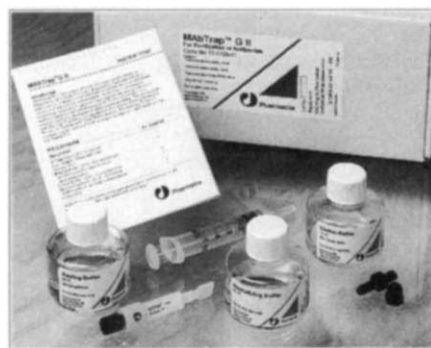
endonuclease and exonuclease, and protease activity, the reproducibility problems inherent with other yeast lytic enzymes are eliminated. One unit of Quantazyme is equivalent to 20–100 units or more of other yeast lytic enzymes. Stated applications include: genome research, YACs, extraction, yeast transformation, protoplast formation, yeast cell disruption, industrial processes, yeast inactivation, food processing and yeast recycling.

BioCoat cell growth environments from Collaborative Biomedical Products/Becton Dickinson Labware are designed to provide cells with conditions that closely mimic their natural environment *in vivo* (*Reader Service No. 102*). The company is now offering two new products: the BioCoat Endothelial and BioCoat Keratinocyte cell growth environments. These cell culture systems are designed to promote rapid growth of monolayers of cells *in vitro*. Each system integrates specially formulated low-serum or serum-free medium, culture supplements and growth factors, as well as BioCoat Cellware T-75 flasks treated with cell-specific extracellular matrix proteins. The company states that cells grown in these cell culture systems will double at least twice as fast as cells grown in commonly used serum-containing medium: using BioCoat cell growth environments, the cell number will

increase up to eight times over a five-day period from an initial seed density of 5×10^5 cells per 75 cm^2 . Growth of endothelial cells from a variety of sources is supported, including human umbilical vein, pulmonary artery, aorta and fetal bovine heart. The BioCoat keratinocyte growth environment promotes the growth of normal keratinocytes such as human normal epidermal keratinocytes from neonatal foreskin and adult epidermis.

■ Pure genius

MABTrap G II from Pharmacia LKB is designed to purify monoclonal and polyclonal IgG from ascites fluid, serum and cell culture supernatants within 10–15 min using only a syringe or a simple peristaltic pump (*Reader Service No. 103*). The kit comprises



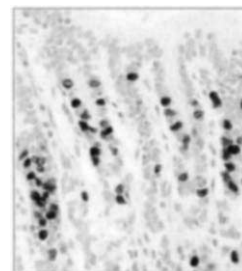
MABTrap G II affinity kit for IgG purification.

a 1 ml HiTrap Protein-G column, three pre-made buffer concentrates, a syringe and Luer adaptor. Also included are instructions on how to prepare buffers and samples, and examples of purification protocols. Enough reagents are provided to perform up to 20 purifications. The method is designed to be quick and easy: take the Protein-G column and equilibrate it, load the sample, wash out unbound material, and elute the purified IgG using the buffers and syringe supplied.

The E-Z-SEP product line from Middlesex Sciences offers researchers a novel antibody purification method that provides purified, immunologically active antibodies in a quick 'mix-spin-resuspend' format. Now this simple liquid polymer separation technique has been applied to even more 'specialized' purification needs (*Reader Service No. 104*). With the company's Bound/Free reagent, free label can be removed from antibody-label conjugate preparations, while the yield and activity of the conjugate is maintained. This universal reagent removes labels of all types: enzyme labels, radiolabels, fluorophores and chromophores. In addition, Middlesex Sciences has introduced a simple and efficient method to delipidate and purify IgY, chicken IgG, from egg yolk. The method requires no complex delipidation procedures and uses no organic solvents. The gammaYolk reagent, a liquid formulation of polymers, is designed to provide high yields of purified, immunologically active antibodies following a simple centrifugation procedure.

■ Kits

Zymed is offering a new kit for staining BrdU, in cell proliferation studies, using frozen or paraffin-embedded cell culture and tissue samples (*Reader Service No. 105*). The kit optimizes antigen presentation in the sample and uses DAB as a chromogen. It can be used with samples from any species, including mouse, rat, rabbit and human, without background staining. The kit includes high-affinity monoclonal anti-BrdU, universal blocking reagent, positive



Zymed's BrdU immunostaining kit.

control slides, denaturing solution, tissue digester, DAB detection reagents, counterstain and mounting solution.

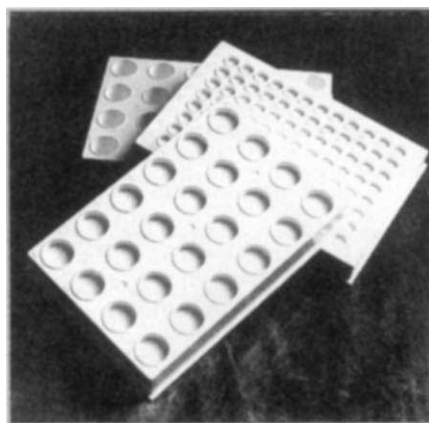
Using the QIFIKIT quantitative immunofluorescence indirect assay from Biocytex, which is based on the use of microbeads mimicking monoclonal antibody-bearing cells, a comprehensive series of **flow cytometry standards** allows absolute quantitation of antibody binding to cell surfaces and antigen/receptor expression (*Reader Service No. 106*). With QIFIKIT, users of flow cytometry are able to move from arbitrary units of fluorescence to antigen density (sites per cell). In this way, it is possible to compare immunofluorescence results over time and between laboratories. Needing only crude sources of unconjugated monoclonals, and adapted to indirect staining protocols, the QIFI approach is designed to provide a cost-effective means of quantifying adhesion molecules, activation antigens and tumour markers.

QUICKMeasure from Sterogene is intended to be a fast and convenient way of determining the **concentration of monoclonal antibodies in cell culture or during purification** (*Reader Service No. 107*). The kit can be used to determine the concentration of any class or subclass of antibody, including IgG, IgM, IgA and IgE in about 30–60 min. If serum supplements are used in the culture medium, Sterogene says that bovine Ig does not interfere with QUICKMeasure. The kit can be used to optimize cell culture conditions and to monitor/optimize purification conditions.

Assays

The new single component TMB peroxidase enzyme immunoassay (EIA) **substrate kit** from Bio-Rad is designed to provide the user with greater convenience and speed than conventional TMB reagents in microplate analysis, but not at the expense of sensitivity or stability (*Reader Service No. 108*). Bio-Rad says that TMB is the preferred EIA substrate for detecting antibodies labelled with horseradish peroxidase in kinetic and endpoint microplate assays.

Packard has introduced **24-well microplates** for tissue culture and in-plate binding assays (*Reader Service No. 109*). These microplates are often used in place of 96-well plates for biological assays that require larger amounts of material to achieve a signal that is sufficient for quantitation. The microplates complement the company's TopCount scintillation and luminescence counter: as the same microplates can be analysed directly in TopCount, a simple wash step is all that is required for separating bound from free label. CulturPlate-24 and ViewPlate-24 are new 24-well microplates for tissue culture and adherent cell growth. The former is a white, opaque, polystyrene plate that is tissue-



Packard's 24-well microplates for assays requiring high sensitivity.

culture treated, individually wrapped with a lid and sterilized. The white reflective polystyrene is designed to prevent optical cross-talk for multidetector counters such as TopCount. The ViewPlate-24 is similarly treated and packaged, but differs from the CulturPlate in that it has a clear, transparent bottom that allows for microscopic observation of cell growth and morphology before analysis. The OptiPlate-24 is an untreated polystyrene plate designed for large surface area in-plate assays or large volume (2-ml well capacity) liquid scintillation counting. The procedures for coating polystyrene with bovine serum albumin, biotin, streptavidin and other combinations are all applicable to OptiPlates.

A new brochure available from Millipore describes the company's **Multi-Screen assay system** (*Reader Service No. 110*). Millipore says that the system offers advantages over conventional techniques like ELISAs or cell harvesters when used for routine research assays and drug discovery techniques. A step-by-step explanation of how the system operates, from application of the sample to quantitation, is provided. The performance of Multi-Screen's filter-based, 96-well format plates over other methods is described. Users can also re-request information on a range of proven applications.

Advanced Magnetics has introduced the TiterZyme murine interleukin-6 (IL-6) sandwich enzyme immunoassay (EIA) kit for the **quantitative determination of mouse IL-6 levels** in serum or cell culture supernatant (*Reader Service No. 111*). Interleukin-6, also known as interferon- β 2, has been shown to play a critical role in immune defence mechanisms. Precision wells, coated with murine IL-6, are designed to provide both consistency and flexibility. In addition AMI says



Millipore's MultiScreen.

that prediluted, lyophilized standards eliminate dilution variability. The EIA has been optimized for speed and sensitivity: assay results can be obtained in three hours and the stated sensitivity is 7.0 pg ml^{-1} in buffer and serum diluent. An optional room temperature shaking format is offered to increase sensitivity to less than 1.0 pg ml^{-1} in buffer and serum diluent. Interleukin-6 is specific for bioactive mouse IL-6. The kit contains sufficient reagents for 96 tests, including standard curve.

The ZDV-Trac **AZT radioimmunoassay kit**, the joint effort of Incstar and Burroughs Wellcome, has, says Incstar, proven to be sensitive enough to conduct intracellular research and specific as well as accurate enough to conduct exacting pharmacological research with other drugs in combination with AZT (*Reader Service No. 112*). Besides accessing AZT levels, the protocol instructs the user on a method of measuring GAZT levels. Incstar says that researchers have used the technology to access levels of AZT to humans, dogs, cats, rats, mice, monkeys and other species. The radioimmunoassay technology is based on the isotope ^{125}I , which eliminates the need to deal with counting solvents associated with beta-counting methods.

Assorted antibodies

The extracellular matrix (ECM) is a key component in healthy cell structures. Invasion and metastasis in cancer diseases are a complex phenomenon in which tumour cells interact as a result of host factors such as immune response and extracellular matrix. Detection of extracellular matrix components in various parts of the body provides an efficient means of following and describing metastasis. Biohit has now launched a new range of **monoclonal antibodies to extracellular matrix components** to complement its range of human integrins, cytoskeletal polypeptides, neurotransmitter substances and endothelial cell surface markers (*Reader Service No. 113*).

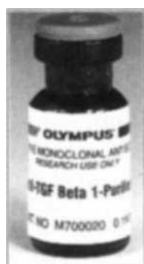


ECM monoclonals.

Several new mouse, rat and human monoclonal antibodies have been added to Endogen's line of **antibodies to adhesion molecules and cell surface markers** (*Reader Service No. 114*). The new antibodies include: anti-human P-selectin (CD62), anti-mouse ICAM-1 (CD54), anti-mouse and anti-rat β 2 integrin (CD18), anti-rat NKRP-1, anti-rat LFA-1 α (CD11a), anti-rat VLA-4 α (CD49d), anti-rat cell-CAM 105 (C-CAM), and anti-rat dipeptidyl peptidase IV (CD26). The antibodies are available in a purified, sterile, carrier- and additive-free formulation. They are suitable for use in functional assays, immunoprecipitation, im-

munohistochemistry and western blots. For researchers interested in multicolour analysis by flow cytometry, Endogen also provides antibody directly labelled with biotin or fluorescein.

The immunochemicals department of Olympus America now offers a **monoclonal antibody reagent for human transforming growth factor beta-1** (TGF beta-1) (*Reader Service No. 115*). The reagent can be used for functional neutralization, western blot analysis, and paraffin-embedded tissue staining. Anti-TGF beta-1 is supplied as a purified monoclonal antibody, free of preservatives and extraneous protein.



TGF beta-1 antibody.

AMAC, a subsidiary of Immunotech has introduced two new monoclonal antibodies: **progesterone receptor antibody and p53 monoclonal antibody** (*Reader Service No. 116*). AMAC's PR10A9, IgG2a, recognizes a dodecapeptide of the C-terminal sequence of the human progesterone receptor expressed in normal breast and uterine tissue and in breast and endometrial cancer tissues. The antibody, clone DO1, IgG, recognizes a fixative-resistant epitope on the N-terminal 45-amino acid fragment of human p53 protein in malignant colon, bladder, breast and lung tissue, as well as melanomas. It reacts with both the wild-type and mutant forms of p53. Both antibodies can be used on routinely fixed, paraffin-embedded tissue sections processed by microwave technique and are suitable for use in flow cytometry and immunohistochemistry staining of frozen sections without pretreatment. They are supplied as pre-diluted 6-ml aliquots and are stable for 12 months at 2-8 °C.

Two new **cyclic nucleotide antibodies** suitable for radioimmunoassay (RIA) are now available from Calbiochem (*Reader Service No. 117*). Anti-cyclic AMP, rabbit, can be used for cAMP RIAs and adenylylcyclase assays. The stated sensitivity is less than 0.5 pg per 50 µl in the acetylated cAMP. Calbiochem says that no detectable displacement from total binding was produced by ATP, ADP, GTP, GDP or cGMP. Anti-cyclic GMP, rabbit, on the other hand, can be used for cGMP RIAs. The sensitivity is said to be less than 0.5 pg per 50 µl in the acetylated cGMP RIA and this antibody does not crossreact with cAMP, ATP, ADP or AMP up to a concentration of 1 mM.

Advanced Biotechnologies announces the availability of **polyclonal antibodies to three drugs of abuse**: phencyclidine (PCP), tetrahydrocannabinol (THC) and temazepam (*Reader Service No. 118*). PCP is known to be a potent analgesic anaesthetic and is used

as a drug of abuse because of its hallucinogenic properties. THC is one of the active constituents of marijuana prepared from the leaves and flowers of *Cannabis sativa*. Temazepam is a member of the benzodiazepine group of compounds which are widely abused because of their sedative hypnotic effects. All three antibodies were raised in sheep and have been assessed by PFIA.

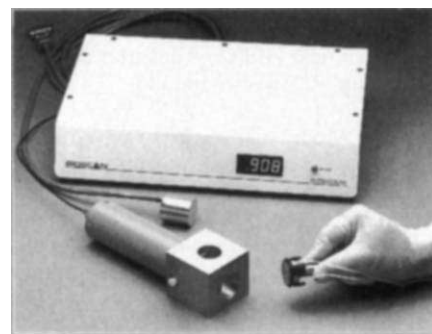
PharMingen introduces several new monoclonal antibodies for human immunology research. For **research in apoptosis**, the company now has monoclonal antibodies specific for Fas antigen (APO-1), *bcl-2* and CD40 (*Reader Service No. 119*). Anti-human Fas mediates apoptosis and can be used for studies of *in vivo* activation status, clonal deletion, autoimmune diseases and cell-mediated cytotoxicity. Anti-human *bcl-2* should be a useful tool for researching the role of *bcl-2* in human follicular lymphomas, as well as resistance to apoptosis. Lastly, anti-human CD40 induces B-cell stimulation through reaction with its ligand. Stated applications include analysis of T- and B-cell interaction, B-cell activation, and the role of CD40 in prevention of apoptosis. Anti-human Thy-1 is also available from PharMingen. Expression of human Thy-1 has been found to be highest on a subset of primitive haematopoietic progenitor cells. It can be used for studies in human haematopoiesis, stem-cell biology and unique lymphoid subsets. In addition, PharMingen provides a complete line of monoclonal antibodies against cell-cycle-associated proteins. Now available are monoclonal antibodies specific for human p34, cdk2, PCNA, GAP, MAP2 kinase, EGF receptor, and cyclins A, B1, D1, D2, D3, pan-D and E. Reagents for characterization of transcription factor-associated proteins E2-2, E12

and E47 have also been released. Monoclonal anti-*fos* protein detects both mouse and human *c-fos* in western blots.

Affinity BioReagents offers a comprehensive line of **stress response antibodies** including HSP70, HSP90 and HSP104 (*Reader Service No. 120*). New additions to this line include two monoclonals to human mitochondrial HSP60. ABR's new GRP78/BiP-specific polyclonal immunoprecipitates GRP78 in murine systems. If KDEL proteins are of interest, the company offers a new polyclonal that recognizes GRP78, GRP94 and PDI.

Hardware

Bioscan has developed a new line of **radioactivity flow counters** specifically for monitoring column effluents in the preparative and analytical HPLC of radiolabelled mono-



Flow-Count — a radiochromatography flow monitor for monoclonals and PET.

clonal antibodies (*Reader Service No. 121*). Applications include research, development and quality control of radiopharmaceuticals for the treatment of cancer and for metabolic imaging. Flow-Count provides remotely-operated, user-selectable detectors, which

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PRODUCT REVIEW

are separate from the base electronics unit. These space-saving, interchangeable detectors fit into tight spaces in the hood and cover a wide range of activities from 100 d.p.m. to 1 Ci. Quality control of radiolabelled monoclonal antibody by analytical HPLC is performed with scintillation detectors optimized for gamma and beta isotopes such as ^{111}In , $^{99\text{m}}\text{Tc}$, ^{90}Y , ^{131}I , ^{125}I , ^{123}I , ^{124}I , ^{67}Ga , ^{212}Bi , ^{186}Re and ^{188}Re . Several versions are available to detect from 10^2 to 10^8 d.p.m. In addition, for very high-activity samples, such as preparative HPLC column effluents, Flow-Count has a miniature, solid-state detector option with an activity range of 10 μCi up to almost 1 Ci. Use of Flow-Count in conjunction with an ultraviolet detector allows overlay of UV and radioactivity peaks to ensure that they match and the product is pure. Flow-Count's analog output signal is compatible with all recorders, integrators and chromatography data systems. For clinical PET centres, Flow-Count detectors are available for preparative and analytical HPLC of positron emitters, including ^{11}C , ^{18}F , ^{13}N and ^{15}O .

The Model C85-5 **low-temperature laboratory freezer** from So-Low is designed for a myriad of uses in medical research: biological storage, preserving tissue, plasma, blood cells and for microbiological applications (*Reader Service No. 122*). The cabinet has an all-steel construction with a door that opens frontwards. A temperature control provides an adjustable temperature range from -18°C to -85°C . The cabinet has an air-cooled condenser, so no liquid is required. The freezer offers 5-cubic feet of storage space, chamber dimensions of $30 \times 18 \times 16$ inches and outside dimensions of $40 \times 35 \times 46$ inches.

The Shandon Consul **automatic coverslipper** from Life Sciences International now has several enhancements (*Reader Service No. 123*). Included among these is a new computerized, high precision mountant dispensing system that is designed to ensure that precise volumes of mountants are dispensed, consistently and repeatedly, with one syringe to suit all routine smears and sections. The mountant reservoir bottle also



Shandon's Consul automatic coverslipper.

incorporates an easy-to-use filler port for topping up the mountant. The operational routine has been simplified to four steps: replenishing mountant, selecting de-gas (to remove bubbles from mountant), loading coverslips and slides, and pressing start to commence the cycle. Efficiency and throughput can be further maximized by integrating the Consul with one of Shandon's automated stainers such as the Varistain.

The new **range of vessels** for LH Fermentation's Discovery 210 bioreactors can be steam-sterilized *in situ* (*Reader Service No. 124*). This feature eliminates the need for disassembly, or for an autoclave. The vessels are available in 5-, 7-, 10-, 15- or 20-litre versions; all have twelve 19 mm top-plate ports, although the large 10-, 15- and 20-litre vessels come complete with five 25 mm side-entry Ingold-style ports for DO_2 , CO_2 , pH, temperature or for the measurement of cell density. A choice of direct or magnetic drive options is available to suit either animal cell or microbial cultures.

■ Shelf stockers

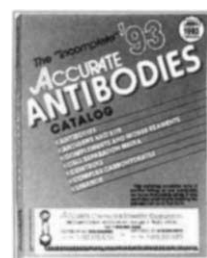
The 1993 Chemicon catalogue lists new **reagents for use in environmental compounds testing or research** (*Reader Service No. 125*). The company offers a line of polyclonal antibodies, antigens and antigens labelled with horseradish peroxidase to environmental compounds. Available anti-

bodies include anti-alar, atrazine, carbofuran, heptachlor, malathion, sulphamethazine and toluene. Antigens available include alar, atrazine, carbofuran, heptachlor, sulphamethazine and toluene.

A new **catalogue of immunochemicals** is now available from ImmunoGen (*Reader Service No. 126*). The company's product range features polyclonal antibodies for OEM and research applications, antibodies to drugs and other low-molecular-weight haptens, a range of primary antibodies to hormones, steroids, proteins and infectious disease agents, secondary antibodies, monoclonal antibodies, drug derivatives and conjugates.

A new 370-page **catalogue**, containing over 20,000 entries, is now available from Accurate Chemical & Scientific (*Reader Service No. 127*).

The catalogue includes a line of antibodies, antigens and kits — one-step cell separation media, from antilymphocytes sera production to fractionation of viruses in routine and research applications — complex carbohydrates — ion channel and receptor ligands — and coagulation products and controls.



Accurate Scientific's antibody selection.

T Cell Diagnostics introduces its 1993 **research products catalogue**, which highlights its comprehensive cell-surface marker and cytokine enzyme immunoassay test kits and T-cell receptor, adhesion and activation monoclonal antibodies (*Reader Service No. 128*). The catalogue should provide a useful technical resource as it offers discussions of many immune molecules and their expression in various conditions. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal.

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